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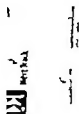
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Measurement of cerebral blood flow in the fetal lamb with a note on the flow-distribution

By

SVANTE BLONSTRAND, KIRSTEN KARLSSON and INGEMAR KJELLMER

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Abstract

BLONSTRAND, S., K. KARLSSON and I. KJELLMER. Measurement of cerebral blood flow in the fetal lamb—with a note on the flow-distribution. *Acta physiol. scand.* 1978. 103: 1-8

The cerebral blood flow was measured in the acutely exteriorized fetal lamb by ^{133}Xe on washout and microsphere distribution techniques. The measurements were performed at different blood gas levels. Regional cerebral blood flow was calculated from the microsphere distribution for five different parts of the brain. The gross estimates for blood flow in both the grey and white matter of the hemispheres, which were in close agreement with the cerebral blood flow estimated by the ^{133}Xe on washout technique. The microsphere distribution shows that the fetal cerebral hemisphere has low blood flow compared to the basal area of the brain, and that this difference is increased during hypoxia and hypercarbia.

Key words: Cerebral blood flow. Fetus, ^{133}Xe on (washout), Microspheres (distribution). CBF: Cerebral blood flow; rCBF: Regional cerebral blood flow.

In previous studies we have used the ^{133}Xe on washout technique for estimating the blood flow of cerebral cortex in the acutely exteriorized fetal lamb (Karlsson *et al.* 1974; Kjellmer *et al.* 1974). Purves and James (1969) thoroughly discussed the limitations and applicability of this method. They used local microinjections of ^{133}Xe on in grey or white matter of cerebral cortex in fetal lambs to validate the method of resolving the washout curve into two phases—one fast and one slow phase. Their conclusions were that these two phases corresponded to grey and white matter clearance rates and thus permitted the calculation of grey and white matter blood flow. Although they were unable to recover any recirculating isotope, the very organization of the fetal circulation with 1/3-1/2 of the combined cardiac output traversing the umbilical flow circuit, while the remaining flow bypasses the placenta, necessitates proportion of tracer recirculating to the brain. Thus the slow phase most plausibly in part will reflect recirculation of ^{133}Xe on.

We therefore used only the fast phase of the clearance curve to calculate the CBF. At basal blood gas levels this was estimated to 0.5-0.7 ml/g min⁻¹ (Kjellmer *et al.* 1974). This is in close agreement with the values reported by Purves and James (1969), as is the rise to

two to threefold values when the animal is exposed to low oxygen and/or high carbon dioxide tensions.

Rudolph *et al* (1970) and Makowski *et al* (1972) using the microsphere distribution technique in the chronically instrumented lamb fetus, arrived at considerably higher estimates of CBF i.e. 0.9–1.2 ml/g min at basal conditions. Several possible explanations present themselves to account for this discrepancy. The ^{133}Xe method might underestimate blood flow: the blood flows may be truly different because of the difference between the arterialized preparation and the unanaesthetized chronic preparation or because of differences in blood gas levels. The difference might also be attributed to a grossly inhomogeneous blood flow distribution within the brain, since the flow values derived from the microsphere experiments refer to overall blood flow of the brain while the flow values obtained by the ^{133}Xe technique most probably only reflect cortical blood flow.

The present series of experiments was therefore undertaken to answer two questions:

- 1) Is the blood flow homogeneously distributed to the various parts of the brain at normal and reduced oxygen tensions?
- 2) Does the rapid phase of elimination in the ^{133}Xe washout curve correspond to blood flow in the grey matter of the cerebral cortex?

Material and Methods

10 pregnant ewes with 13 fetuses were investigated. Gestational age, which was either known or estimated from weight and crown-rump length of the fetuses according to the values given by Joubert (1956), and between 120 and 145 days. The ewes were anaesthetized with Ketamin 1–2.0 mg/kg and anaesthesia was maintained with chloralose 35 mg/kg with small doses added hourly. The ewe was tracheotomized and ventilated with known gas mixtures with a pressure-cycled ventilator. Arterial blood gases were used as direct respiratory settings. A slow i.v. infusion of 10% glucose solution was given to maintain the blood sugar at normal levels throughout the experiment.

The fetus was exteriorized and placed on a thermostated table where body temperature and humidity could be maintained. Great care was taken to minimize disturbance of the umbilical circulation. The preparation of the fetus has been described previously (Kjellmer *et al* 1974). It involves cannulating the trachea and connecting it to a saline-filled reservoir cannulating the right brachial artery for blood pressure recordings and arterial blood samples, and measuring deep body temperature with a thermistor in the rectum.

Washout method

The principal arrangement of the preparation is shown in Fig. 1A. The left lingual artery was cannulated in the retrograde direction with polyethylene tubing, PE 90, for the injection of ^{133}Xe saline into the carotid artery. Gamma radiation was recorded over the skull with a thallium-activated scintillation detector, diameter 5 cm. The detector was filled in a lead cylinder 10 cm from the bottom, where a lead diaphragm with a central hole 2 cm in diameter was shielding the detector. This diaphragm was placed 1 cm from the fetal scalp and centered over the left parietal region. The radioactivity was recorded with a ratemeter (type constant 3 s, and written out by a linear potentiometer) and recorder (Servogor Götz Elektro, RE 520, Austria) at a paper speed of 0.5 mm/s. The washout curves were analysed as described previously (Kjellmer *et al* 1974). After background subtraction a semilogarithmic plot was drawn and the slow phase of tracer washout defined. After subtracting this phase from the total curve the resulting rapid clearance phase was obtained and its half-time used to calculate the cortical blood flow.

Microsphere method

In Fig. 1B the principal arrangement of the fetal circulation and of the preparation is shown. The femoral vein was cannulated and a catheter introduced into the inferior caval vein. Through this

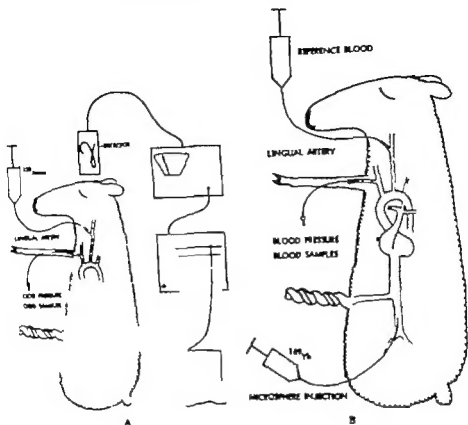


Fig. 1 A schematic presentation of the set up and preparation for determination of CBF by the ^{133}Xe technique (1A) and for determination of CBF by the microsphere technique (1B). In the latter drawing the fetal sheath is also presented.

was of microspheres, diameter 25 μm , tagged with ^{90}Yb was injected. The injection chamber was carefully sparged before and during the injection by high frequency whisker. The injection of approx. 300 000 spheres in 2 ml Dextran solution lasted 15 s, and then the chamber was rinsed by the further injection of 6-10 ml of 6 Dextran solution. The reference blood was obtained by the continuous withdrawal of blood at constant rate (1.50 ml/min) through the catheter in the lingual artery. The withdrawal pump was started 15 s before the injection, and blood was collected for total time of 2 min.

After the experiments had been ended, the brain of the fetus was removed and dissected into 4 different parts, including oblongata and pons, cerebellum, hemispheres, and the rest of the medulla, called basal ganglia. The hemispheres were further divided in white and grey matter. The fresh wet weight of these 5 parts was determined. The radioactivity of the reference blood and of the different parts of the brain was then measured using whole body counter designed for use with small animals (Packard Model 440 Armac Counting System). By measuring the activity in known number of spheres, the total amount of spheres in each specimen could be calculated. According to Buckberg *et al* (1971) at least 400 spheres should be distributed to each specimen to make the estimate of blood flow reliable. This was achieved on all occasions.

The CBF-determinations were made at normoxia and at different levels of hypoxia. The air was ventilated with gas mixture, which gave the desired level of oxygen and carbon dioxide tension in her blood. The fetal CBF was then determined with the ^{133}Xe technique, the injection being given not earlier than 20 min after the respirator gases had been changed. After recording the washout curve for 20-25 min, the

two to threefold values when the animal is exposed to low oxygen and/or high carbon dioxide tensions.

Rudolph *et al* (1970) and Makowaki *et al* (1972), using the microsphere distribution technique in the chronically instrumented lamb fetus, arrived at considerably higher estimates of CBF i.e. 0.9–1.2 ml/g min at basal conditions. Several possible explanations present themselves to account for this discrepancy. The ^{133}Xe method might underestimate blood flow: the blood flows may be truly different because of the difference between the exteriorized preparation and the unanaesthetized chronic preparation or because of differences in blood gas levels. The difference might also be attributed to a grossly inhomogeneous blood flow distribution within the brain, since the flow values derived from the microsphere experiments refer to overall blood flow of the brain while the flow values obtained by the ^{133}Xe technique most probably only reflect cortical blood flow.

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The principal arrangement of the preparation is shown in Fig. 1A. The left lingual artery was cannulated in the retrograde direction with polyethylene tubing PE 50, for the injection of ^{133}Xe in saline into the carotid artery. Gamma radiation was recorded over the skull with a thallium-activated scintillation detector (diameter 5 cm). The detector was fitted in a lead cylinder 10 cm from the bottom, where a lead diaphragm with central hole, 2 cm diameter was shielding the detector. This diaphragm was placed 1 cm from the fetal scalp and centered over the left parietal region. The radioactivity was recorded with a ratemeter (containing 3 A), and written out by a linear potentiometer ink recorder (Servogor Goerz Elektro, RE 520, Austria) at a paper speed of 0.5 mm/s. The washout curves were analysed as described previously (Kjellmer *et al* 1974). After background subtraction a semi-logarithmic plot was drawn and the slow phase of tracer washout defined. After subtracting this phase from the total curve, the resulting rapid clearance phase was obtained and its half-time used to calculate the cortical blood flow.

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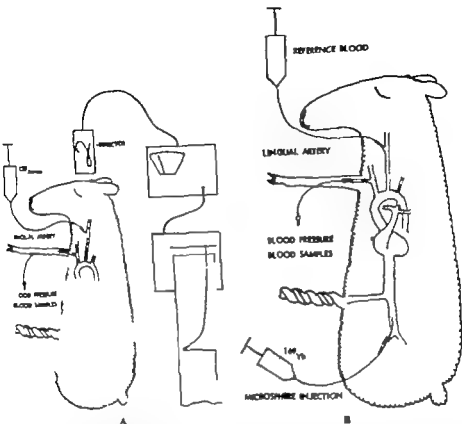


Fig 1 A schematic presentation of the set up and preparation for determination of CBF by the ^{133}Xe ion technique (IA) and for determination of CBF by the microspheres technique (IB). In the latter drawing the fetal elements are also presented.

was of microspheres, diameter 25 μm , tagged with ^{133}Yb was injected. The injection chamber was carefully agitated before and during the injection by high frequency vibrator. The injection of approx. 300 000 spheres in 0.2 ml Dextrose solution lasted 15 s, and then the chamber was closed by the further injection of 6-10 ml of 6 Dextrose solution. The reference blood was obtained by the continuous withdrawal of blood at constant rate (1.50 ml/min) through the catheter in the lingual artery. The withdrawal pump was started 15 s before the injection, and blood was collected for total time of 2 min.

After the experiments had been ended, the brain of the fetus was removed and dissected into 4 different parts, medulla oblongata and pons, cerebellum, hemispheres, and the rest of the medulla, called basal ganglia. The hemispheres were further divided in white and grey matter. The fresh wet weight of these 5 parts was determined. The radioactivity of the reference blood and of the different parts of the brain was then measured using whole body counter designed for use with small animals (Packard, Model 440 Armac Counting System). By measuring the activity in known number of spheres, the total amount of spheres in each specimen could be calculated. According to Brockberg *et al.* (1971) at least 400 spheres should be distributed to each specimen to make the estimate of blood flow reliable. This was achieved on all occasions.

The CBF-determinations were made at normoxia and at different levels of hypoxia. The ewe was ventilated with gas mixture, which gave the desired level of oxygen and carbon dioxide tension in her blood. The fetal CBF was then determined. In the ^{133}Xe ion technique, the injection being given not earlier than 20 min after the respiratory gases had been changed. After recording the washout curve for 20-30 min, the

TABLE II. Linear regression constants.

CBF _{microsphere}	- a	+ b(CBF _{X_{mean}})	r
Cerebellum	- 61	2.81	.86
Pons + Medulla	- 1.87	4.82	.85
Basal ganglia	- 1.52	3.71	.84
Hemisphere, white	- 14	1.23	.86
Hemisphere, grey	- 35	1.43	.86
Total brain	- 55	1.93	.87

The constants *a* and *b* in the equation above, which define the regression lines shown in Fig. 3 B, are given in this table together with the coefficient of correlation, *r*, for the different lines. The equation is valid for $.51 < \text{CBF}_{\text{Xmean}} < 1.10$.

parts of the brain are much steeper than for the hemispheres. In Table II the equations for these lines are given together with their *r* values. Thus there exists a close correlation between the rCBF for all the regions obtained by the microsphere technique and the CBF_{Xmean} but it is also apparent from Fig. 3 B that blood flow in the basal parts of the brain is higher than in the hemisphere, in particular during hypoxia.

Discussion

The rCBF in the white and grey matter of the hemisphere were not significantly different in the present series of expts. This is in contrast to earlier reports in other experimental conditions. Reivich *et al* (1969) using an autoradiographic technique in the adult cat demonstrated quite marked differences between the blood flow in the cortex and in the marrow of the hemisphere, with a higher flow in the cortex, and so have Kennedy *et al* (1972) using the same technique in the newborn dog. The failure in our series of expts. to document a difference between grey and white matter blood flow may be due to too crude a separation between grey and white matter in our preparation. It may also reflect difference due to anesthesia. We have chosen Ketamine instead of barbiturates to induce the anesthesia because of the marked reduction of cerebral oxygen uptake caused by the latter and hence a possible effect on CBF. Chloralose was used for maintenance since it has been regarded as "the ideal anesthetic agent for the study of cardiovascular reflexes" (Grenshelmer 1965). Our earlier experiences with a similar preparation have shown the fetus to have a measurable cortical electrical activity during this anesthesia and at these blood gas levels (Karlsson *et al* 1974). However the functional state of the cortex must be depressed by the anesthesia, and this might contribute to a less pronounced difference in blood flow between grey and white matter compared to awake animals. Speculatively it may also reflect a functional state of the cerebral cortex during fetal life with a low oxygen consumption and a low blood flow in a poorly developed rather inactive neuronal part of the cortex and a metabolically relatively active, myelinating white matter. In Table III our estimates of rCBF in the fetal lamb are compared to other materials, mostly from adult animals. The highest blood flow in the adult animal is found in the cerebral cortex (Reivich *et al* 1969, Foreman *et al* 1976, and Marcus *et al* 1976), while in the newborn dog the highest flow is found in thalamic

TABLE III. Regional cerebral blood flow in different species. A comparison between the present study and data from the literature.

Author	Present study	Kennedy <i>et al.</i> 1972		Reivich <i>et al.</i> 1969	Foreman <i>et al.</i> 1976	Martin <i>et al.</i> 1976
Species	Fetal lamb	Newborn dog	Adult dog	Adult cat	Adult pig	Adult dog
Condition	Anaesthetized	Awake	Awake	Awake	Awake	Awake
Technique	Microspheres	Auto-radiography	Auto-radiography	Auto-radiography	Micro-spheres	Micro-spheres
Measurements of rCBF from						
Hemisphere, grey	47	30-40	45-80	74-122	60	75
Hemisphere, white	40	08-30	10-25	31	41	30
Cerebellum, grey	—	30-40	35-68	83	36	—
Cerebellum, white	—	—	—	24	33	—
Cerebellum, total	1.08	—	—	—	—	66
Basal ganglia	73	40-50	70-105	54-106	43	45
Pons and medulla	90	—	—	—	30	47

All determinations of rCBF were made during normoxia, and in the present study this means the 5 fetuses with arterial pO_2 2.39 kPa. The rCBF is measured in $ml \cdot g^{-1} \cdot min^{-1}$.

nuclei and in cochlear and vestibular nuclei (Kennedy *et al.* 1972). In the newborn dogs the differences between the highest and lowest rCBF are small compared to the corresponding differences in the adult dogs. They were also able to demonstrate that the blood flow to different structures in the brain rose from a comparatively low level in the newborn puppy to a peak level at an age of 3-8 weeks and then declined to the adult level. The peak levels of blood flow occur during the time when the myelination is most intense in a region. It is tempting to suggest that the distribution of flow in the fetal brain, which is quite different from the pattern in adult animals, is explainable along the same lines.

The differences between our earlier determinations of CBF using the ^{133}Xe washout technique (Kjellmer *et al.* 1974) and others using the labelled microsphere distribution technique (Radolph *et al.* 1970, and Makowski *et al.* 1972) can now be explained by the present findings. The ^{133}Xe washout technique measures the blood flow in the cerebral hemisphere accurately but this region in the fetal brain has a lower blood flow than the basal cerebral structures. The microsphere technique used to measure overall cerebral blood flow will thus give a higher value, and the difference between the two methods will be greater during hypoxia or hypercarbia with a higher blood flow which is apparent from the equation for the regression line for the whole brain in Table II.

We are obliged to civil engineer Åke Cedersblad, Dept. of Nuclear Physics, Sahlgrenska Hospital, for his kind help with measurements of the activity from the microspheres. This study was supported by Medical Research Council (Grant 19X 2391) and by Fritsbergstiftelsen, Göteborg.

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The effects of Triton-detergents on the stretch receptor of the crayfish

By

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Abstract

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The effects of the non-ionic detergents Triton X-43 and Triton X 100 on the action potential and the receptor potential of the stretch receptor neuron of the crayfish *Aspidea flaccidula* was studied with intracellular recording techniques. Membrane currents were measured with voltage clamp technique. Both detergents blocked the action potential in 20-30 min at concentrations of 60-80 μ M. Following blocking of spike electrogenesis the receptor potential evoked by stretch was obtained in isolation. With prolonged exposure of the neuron to the detergent there was slowly developing reduction of the receptor potential and after 60-90 min no response to stretch could be obtained. These effects were produced without any significant change of the resting membrane potential. Following return to normal saline the responsiveness to stretch was completely restored in 80-100 min. Measurements with voltage clamp technique showed that the passive membrane properties were little affected by the two detergents. The stretch induced current on the other hand was severely depressed and almost abolished with prolonged exposure. The experimental results suggest that non-ionic detergents block the spike electrogenesis and the transducer action by selective action on the sodium channels of the membrane of the receptor neuron.

It has been reported that non-ionic detergents have an action on peripheral nerves and muscle closely like local anesthetics (Soeborg, Dain and Stave 1952, Zipf and Dittmann 1964, Dittmann 1973). The mechanisms underlying the blocking effect are unknown. Recent observations by Baumær and Rydqvist (1978) suggest, however, that non-ionic detergents have selective action on the sodium channels of the nerve membrane. This observation raised the question whether or not these substances also blocked the activity of peripheral receptors.

It is known from studies on different kinds of sensory organs that the properties of the receptor membranes differ in many respects from that of membranes producing action potential. The main portion of the current induced in transducer membranes during activity is carried by sodium ions but there is also an unknown amount of contribution of other ions. These currents are resistant to the action of many substances like tetrodotoxin (TTX) and local anesthetics.

The present study was undertaken in order to examine the action of two members of the Triton series with different lipophilicity on the crustacean stretch receptor organ. The dendrites of this organ are sensitive to stretch of the muscle to which the neuron is attached. The cell is relatively large and this makes it possible to measure the membrane current induced by a given stimulus with voltage clamp technique. The stretch receptor neuron therefore appeared to be suitable as a model for the study of the action of the detergents on the receptor membrane. The results show that Triton X-45 and Triton X 100 have a blocking effect on the impulse generating membrane of the neuron. With prolonged exposure the activity of the transducer membrane was reduced and finally abolished. There was no appreciable change of the resting membrane potential accompanying the blocking action. It is suggested that the observed effects are due to an action mainly on the potential dependent sodium permeability mechanism of the impulse generating membrane as well as of the transducer membrane of the neuron.

Methods

The experiments were carried out on the slowly adapting stretch receptor of the crayfish (*Decapoda*). The receptor neuron together with its muscle were isolated from the second or third abdominal segment and mounted in a small chamber filled with saline of the following composition in mM: NaCl 226, KCl 5.4, CaCl_2 13.5, MgCl_2 1.2 (van Harreveld 1936) and was buffered with 10 mM Tris to pH 7.2–7.4 (23°C). The temperature of the bath was kept at +15°C.

The receptor muscle was tied at each end to a thin metal rod (0.1 mm), each rod being attached to a Brush pen motor with position and velocity feed-back. The position transducer signal was used to record displacement. Stretch was applied by symmetrical extension of the receptor muscle towards its both ends. The parameters of the applied stretch were varied by driving the pen motors with electrical pulses of different wave forms.

The receptor neuron was penetrated with two microelectrodes, one being used to record the membrane potential, the other to pass current. Both electrodes were filled with 3 M KCl, and had a resistance in the range of 5–10 MΩ. The membrane potential was recorded with a Mentor N 950 preamplifier from which the signals were fed into a tape-recorder (Hewlett Packard 3960) with passband 0–1250 or 0–5000 Hz before being displayed on a Tektronix 5321 oscilloscope. The output of the Mentor amplifier was connected to a clamp amplifier with a maximum output of ± 150 V. The loop gain was set to values between 5000 to 8000 depending on the resistance of the microelectrodes (Brown, Ottoson and Rydqvist. To be published). Membrane current was measured across a feed-back resistor of an operational amplifier used to maintain the bath at ground potential via an Ag-AgCl-electrode. The measurements of the membrane current were made with the receptor muscle kept at resting length and during the application of brief (about 1 ms) ramp and hold extensions. The membrane potential in these experiments was gradually shifted to a positive potential value of about +40 mV from this value the potential was moved in steps of about –10 mV.

The current measurements were made at a time when the membrane current had stabilized at the new level which usually occurred 30 s after application of the command pulse.

The rate of rise of the action potentials was measured by differentiating signals through a Miller-differentiator and was displayed together with the action potentials on the oscilloscope screen.

The effect of Triton X-45 and Triton X 100 were tested in preliminary experiments to find out the time course of their action at different concentrations. In the present study concentrations between 40 and 80 μM were chosen since at these concentrations the blocking effect developed at a time course which allowed for carrying out the current measurements. Triton X-45 and Triton X 100 were gifts from Rohm and Haas Co. and used without further purification. These substances belong to a series of homologous polyoxyethylen-p-4-octyl-phenols with varying number of oxyethylene groups. Triton X-45 has an average number of 4.5 oxyethylene units and a molecular weight of 426. Triton X 100 has an average number of 9.6 oxyethylene units and a molecular weight of 628 (Rohm and Haas, CS-40 1/2 and CS-01a, see also Simons, Helenius and Garoff 1973). The HLB numbers (hydrophilic lipophilic balance number) for Triton X-45 and Triton X 100 are 10.4 and 13.5 respectively.

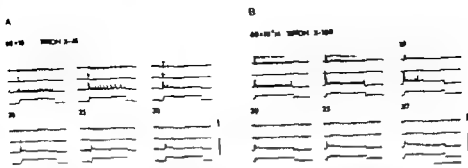


Fig. 1 Impulse responses of crayfish stretch receptor to step-like stretch before and during exposure to A 60 μ M Triton X-43 and B: 60 μ M Triton X 100 for 30 min. Numbers above each recording represent time in min after application of detergent. Top trace: differentiated potential signal, calibration 200 V/s. Second trace from top: zero potential. Third trace from top: membrane potential change, calibration 100 mV. Bottom trace: stretch in arbitrary units. Time bar: 1 s.

Results

Effect on spike electrogenesis

Exposure of the receptor to Triton X-43 at a concentration of 60 μ M caused a gradually developing block of the impulse response to an applied test stretch (Fig. 1 A). The discharge of impulses decreased in duration so that after 10 min a few spikes only were elicited by the extension and only an abortive response was seen after about 25 min. The abolition of the impulse response was preceded by a decrease in amplitude and rate of rise of the individual spikes. As can be seen in Fig. 2 A the maximum rate of rise (V_A) of the spikes falls slowly with time of exposure. At a concentration of 40 μ M blocking does not occur until after more than 30 min. With higher concentrations (60 μ M) the effect on the spikes develops faster and all signs of impulse generation are generally abolished within 20–25 min. In most experiments blocking was not accompanied by any significant change of the resting membrane potential. The change of membrane potential due to receptor activity (receptor potential) remained after the disappearance of the regenerative spikes. Hence the effects of Triton was similar to that of TTX and local anesthetics like procaine and lidocaine in the respect that they all affect the specific sodium permeability mechanism.

On return to normal solution the discharge due to extension reappeared. Recovery was slow and usually incomplete as can be seen in Fig. 2.

The effect of Triton X 100 was closely similar to that of Triton X-43 (Figs 1 B and 2 B). In general, however, the blocking effect developed more slowly than with Triton X-43 at the same concentration of the two substances. As with Triton X-43 blocking of the impulse discharge was not accompanied by any significant change of the resting membrane potential. Recovery of the impulse response had a slow time course similar to that after exposure of the neuron to Triton X-43.

Effect on the receptor potential

Prolonged exposure of the neuron to the detergents caused a block of the impulse generation and reduction and final elimination of the receptor potential as is illustrated in Fig. 3.

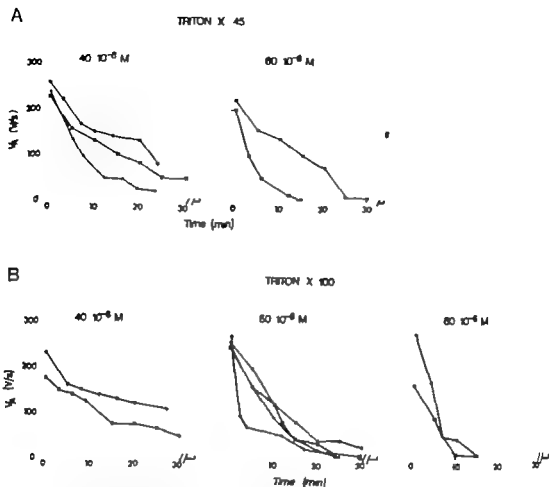


Fig. 2. Maximum rate of rise (V_A) of action potential versus time following exposure of neurons to Triton detergents. Measurements were made over the first 20–30 min and the cells thereafter left for varying periods of time in Triton before being returned to normal saline.

A Triton X-45. In the left hand graph one cell (circles) was exposed for 35 min and recovery in normal saline was measured after an additional period of 130 min. second cell (squares) was exposed for 200 min and recovery measured after 140 min. In the right hand graph one cell (circles) was exposed for 100 min and recovery measured after 140 min. the other cell (squares) was exposed for 25 min and recovery measured after 105 min. *B* Triton X-100. In the left hand graph one cell (circles) was exposed for 70 min but was not returned to normal saline. second cell (squares) was exposed for 3 min and recovery measured after an additional period of 110 min. In the middle graph one cell (squares) was exposed for 35 min and allowed to recover for 100 min. another cell (triangles) was exposed for 120 min and recovery followed for 100 min. The other cells were not returned to normal saline.

After application of Triton X-45 (Fig. 3*A*) the spikes were abolished after 30 min leaving the receptor potential in isolation. Following this there was a gradual slow reduction of the receptor potential and after 90 min exposure of the neuron to Triton X-45 extension did no longer evoke any noticeable receptor response. At this stage the extension had no effect whatsoever on the neuron. The time course of the blocking effect of Triton X-45 on the dynamic and the static phase of the receptor potential is illustrated in Fig. 4. The spikes during static extension of this neuron disappeared after 10 min while the dynamic spike was not

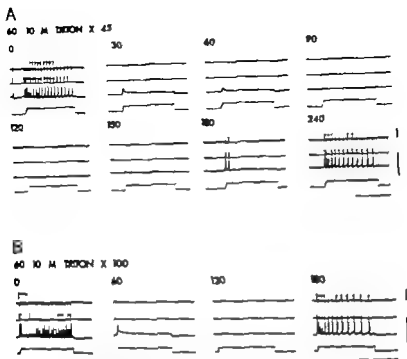


Fig. 3. Impulse and receptor response of crayfish stretch receptor to step-like stretch before and during prolonged exposure to $60 \mu\text{M}$ Triton X-45 (A) and $60 \mu\text{M}$ Triton X-100 (B). Numbers above each recording represent time in min after serial application of detergent. Top trace: differentiated potential signal, calibration 200 V/s. Second trace from top: zero potential. Third trace from top: membrane potential change, calibration 100 mV. Bottom trace: stretch in arbitrary units. Time bar: 1 s. In A the cell was returned to serial saline after 100 min, in B after 170 min.

blocked until after 20 min. Following the block of impulse generation the dynamic and the static phase of the receptor potential remained unaffected for about 30 min. The effect of Triton X-45 on the transducer activity of the neuron thus required an exposure time of about 33 min to develop. Usually the dynamic phase of the response was first affected fol-

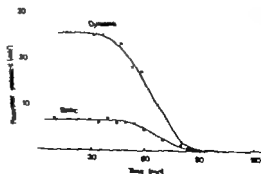
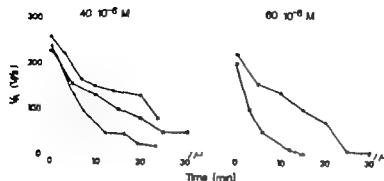


Fig. 4. Effect of Triton X-45 on dynamic and static phase of receptor potential (see text).

A

TRITON X-45



B

TRITON X 100

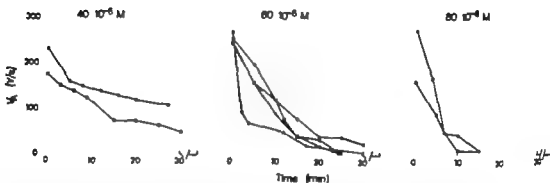


Fig. 2. Maximum rate of rise (V_A) of action potential versus time following exposure of neurons to Triton detergents. Measurements were made over the first 20–30 min and the cells thereafter left for varying periods of time in Triton before being returned to normal saline.

A Triton X-45. In the left hand graph one cell (circles) was exposed for 35 min and recovery in normal saline was measured after an additional period of 130 min. a second cell (squares) was exposed for 200 min and recovery measured after 110 min and a third cell (triangles) was exposed for 100 min and recovery measured after 140 min. In the right hand graph one cell (circles) was exposed for 100 min and recovery measured after 140 min. the other cell (squares) was exposed for 25 min and recovery measured after 105 min. *B* Triton X 100. In the left hand graph one cell (circles) was exposed for 70 min but was not returned to normal saline. a second cell (squares) was exposed for 3 min and recovery measured after an additional period of 110 min. In the middle graph one cell (squares) was exposed for 35 min and allowed to recover for 100 min. another cell (triangles) was exposed for 170 min and recovery followed for 100 min. The other cells were not returned to normal saline.

After application of Triton X-45 (Fig. 3 *A*) the spikes were abolished after 30 min leaving the receptor potential in isolation. Following this there was a gradual slow reduction of the receptor potential and after 90 min exposure of the neuron to Triton X-45 extension did no longer evoke any noticeable receptor response. At this stage the extension had no effect whatsoever on the neuron. The time course of the blocking effect of Triton X-45 on the dynamic and the static phase of the receptor potential is illustrated in Fig. 4. The spikes during static extension of this neuron disappeared after 10 min while the dynamic spike was not

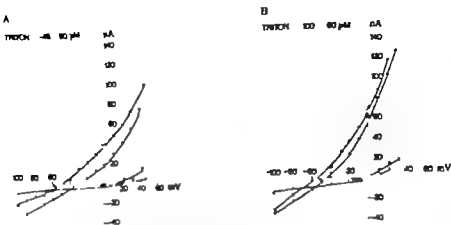


Fig. 6. I-V relations of the steady-state passive currents (solid symbols) and SIC (open symbols) measured as in Fig. 5 before and after treatment of the cell with 60 μ M Triton X-45 (A) and 60 μ M Triton X-100 (B). The neurons were exposed to the detergents for about 60 min.

remained nearly unchanged for more negative potentials. A possible cause for the increased slope conductance and the concomitant decrease in potential at zero current after prolonged exposure to the detergent might be a deterioration of the neuron due to the penetration of two microelectrodes and the repetitive extensions. It was usually not possible to reverse the effect of the detergent in the voltage clamp situation in contrast to measurements with only one micropipette (cf. Fig. 3) or with shorter time of exposure.

Membrane currents induced by stretch. The records in Fig. 5 show examples of the stretch induced current (SIC) during voltage clamp at various membrane potentials before and after application of Triton X-45. The current induced at resting potential was inward and had a configuration and time course closely similar to the potential change induced by the same stretch. The SIC decreased at positive polarization values and disappeared at a membrane potential of 15 mV. At more positive membrane potential values the SIC due to extensions reversed in direction. The time course of the SIC was otherwise unaffected by the membrane potential.

After about 60 min in Triton X-45 there was a marked decrease of the SIC for potential values less than the reversal potential (which was about -20 mV) whereas the SIC appeared to be unchanged or somewhat enhanced for potential values more positive than the reversal potential. After 90 min in Triton X-45 the SIC had diminished still more while the reversal potential remained unchanged (-17 mV).

Fig. 6 shows the I-V relationship of the passive membrane current (solid symbols) and the SIC (open symbols) at different membrane potentials during voltage clamp before and after application of Triton X-45 and Triton X-100. The SIC obtained at each value of E_m is represented by open circles in normal *Astacus* saline.

A comparison between the curves for Triton X-45 and Triton X-100 shows that the effect of Triton X-100 on the SIC was almost identical to that produced by Triton X-45. The changes in the reversal potential varied from one cell to another but was always in the nega-

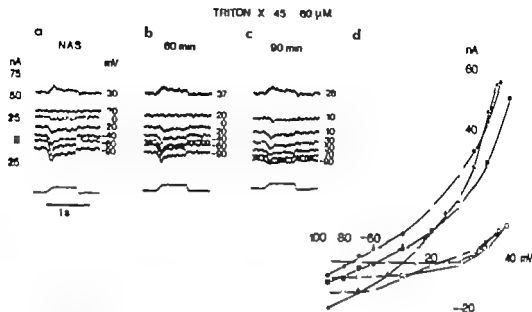


Fig. 5 Receptor membrane currents during rest and stretching in normal saline and after exposure of cell to detergent, Triton X-45. *a-c* Records of SIC (stretch induced current) at different membrane potential levels in normal *A. taeniorhynchus* saline (a), after 60 min (b) and 90 min (c) in 60 μ M Triton X-45. Stretch was applied after the membrane current had reached a steady level. Holding potentials for each recording are shown to the right. *d* *I-V* relations of the steady-state passive current (solid symbols) and SIC (open symbols). SIC was obtained by subtracting the steady-state passive current from the peak membrane current during stretch. Circles: normal *Asterias* saline; squares: after 60 min in Triton X-45; triangles: after 90 min in Triton X-45.

lowed after about 10–15 min by the decrease of the static phase. Complete abolition of the response did not occur until after a total exposure time of about 90 min, the dynamic phase of the response being the last to disappear.

With return to normal solution the dynamic phase of the receptor potential first returned followed later by the dynamic impulse response and still later by the impulses during static extension until the response was almost completely restored. The effect of Triton X 100 on the receptor potential in all respect resembled that of Triton X-45 as can be seen in Fig. 3 *B*.

In summary the effects of Triton X-45 and Triton X 100 consists of an initial blocking of the impulse generating mechanisms of the neuron followed by a later blocking of the transducer action. These effects were produced with small changes of the resting membrane potential.

The effect of detergents examined with voltage clamp

Membrane currents in the resting neuron. As can be seen in Fig. 5 and Fig. 6 Triton X-45 and Triton X 100 have little effect on the passive properties (filled symbols) of the receptor neuron. The cell illustrated in Fig. 5 was exposed to Triton X-45 (60 μ M) for about 90 min. In normal *Asterias* saline (NAS) this cell had a membrane resistance of about 3.7 M Ω at resting membrane potential; this value did not change during exposure to the detergent for 60 min although the cell was polarized to about -40 mV. After 90 min the slope conductance had increased for potentials more positive than the resting membrane potential while it

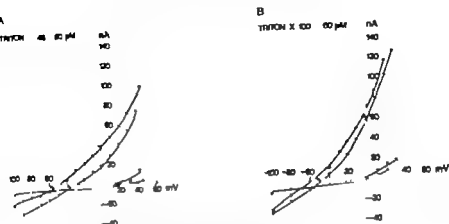


Fig. 6. I-V relations of the steady-state passive currents (solid symbols) and SIC (open symbols) measured as in Fig. 5 before and after treatment of the cell with 60 μ M Triton X-45 (A) and 60 μ M Triton X 100 (B). The neurons were exposed to the detergents for about 60 min.

remained nearly unchanged for more negative potentials. A possible cause for the increased slope conductance and the concomitant decrease in potential at zero current after prolonged exposure to the detergent might be a deterioration of the neuron due to the penetration of two microelectrodes and the repetitive extensions. It was usually not possible to reverse the effect of the detergent in the voltage clamp situation in contrast to measurements with only one micropipette (cf. Fig. 3) or with shorter time of exposure.

Membrane currents induced by stretch. The records in Fig. 5 show examples of the stretch induced current (SIC) during voltage clamp at various membrane potentials before and after application of Triton X-45. The current induced at resting potential was inward and had a configuration and time course closely similar to the potential change induced by the same stretch. The SIC decreased at positive polarization values and disappeared at a membrane potential of 15 mV. At more positive membrane potential values the SIC due to extensions reversed in direction. The time course of the SIC was otherwise unaffected by the membrane potential.

After about 60 min in Triton X-45 there was a marked decrease in the SIC for potential values less than the reversal potential (which was about +20 mV) whereas the SIC appeared to be unchanged or somewhat enhanced for potential values more positive than the reversal potential. After 90 min in Triton X-45 the SIC had diminished still more while the reversal potential remained unchanged (-17 mV).

Fig. 6 shows the I-V relationship of the passive membrane current (solid symbols) and the SIC (open symbols) at different membrane potentials during voltage clamp before and after application of Triton X-45 and Triton X 100. The SIC obtained at each value of E_m is represented by open circles in normal *Astacus salinus*.

A comparison between the curves for Triton X-45 and Triton X 100 shows that the effect of Triton X 100 on the SIC was almost identical to that produced by Triton X-45. The change in the reversal potential varied from one cell to another but was always in the nega-

tive direction for both detergents (towards more negative potential values). In an earlier investigation on the stretch receptor (Brown, Ottoson and Rydqvist to be published) it was observed that the reversal potential appeared to vary with the length and configuration of the main dendrites and that the reversal potential generally was more positive for neurons with a long main dendrite than for neurons with short richly branching dendrites. It is possible therefore that the differences in reversal potential observed might partly be attributed to structural differences of the dendritic tree of the neurons.

Discussion

The present results show that both Triton X-45 and Triton X 100 block the action potential and the receptor potential in the crustacean stretch receptor neuron. At a concentration of 60 μ M the conducted impulses are blocked within 20–30 min and this effect is produced without any appreciable change of the resting membrane potential. It is most likely that the effect on the conducted activity can be ascribed to a block of the potential dependant sodium permeability of the impulse generating membrane as judged from the measurements of the maximum rate of rise of the action potentials. As shown by Hodgkin and Katz (1949) and by Schwartz, Ulbricht and Wagner (1973) this parameter is a good approximation of the sodium current (cf. Rydqvist 1977).

The voltage clamp experiments show that the passive membrane properties *i.e.* the slope conductance was very little affected by the two detergents. In some experiments there was a clear decrease in the slope conductance for potential values more positive than the resting membrane potential. The same effect was seen in preparations soaked in normal *Artemia* saline and appears therefore not to be caused by the detergents. It was also noted in many experiments that the resting membrane potential was reduced after a series of clamping measurements. Following recovery at the resting membrane potential (without being in the clamp-condition) the resting membrane potential usually returned to the pre-clamp values.

As shown Triton detergents cause large reduction of the inward (negative) stretch induced current (SIC). A tentative explanation to this effect is that the detergents have a selective action on the sodium permeability of the receptor membrane. It is probable that the sodium equilibrium potential of the neuron is 40 to 60 mV. A selective decrease of the sodium permeability would therefore diminish the SIC for negative potential values and consequently also the receptor potential. A decrease in sodium permeability of the receptor membrane could be due to a lyotropic interaction between the detergents and the lipid components of the membrane. It is well known that detergents can interact with the lipid bilayer of biological membranes, changing it from the naturally occurring liquid crystal lamellar phase to either a less or more fluid liquid crystalline state or towards the so called gel state (Chapman 1971, Small *et al.* 1966, Small 1968, Wojtowicz 1975). The change to a more stable structure might explain the decrease in sodium permeability. Another possibility is that the detergent reacts with specific sites in the membrane (*e.g.* a protein). In view of the fact that the receptor potential was not affected until after prolonged exposure of the neuron to the detergents this alternative appears less probable at least if it is assumed

that the sites are located at the outer side of the membrane. The slow action on the receptor membrane might indicate that there is an outside diffusion barrier or that the site of action is intracellular.

It is in this context of interest to note that it has been observed in experiments on the motor end plate in frog skeletal muscle that Triton X-100 causes disruption of the membrane of mitochondria (Rydqvist, unpublished observations). It is therefore possible that the effects observed in the present experiments may partly be attributed to an intracellular release of calcium from mitochondria.

The potential at which the SIC reverses direction is invariably changed towards more negative values after treatment with Triton detergents. No appreciable difference between the two detergents was observed. Changes in reversal potential must be due to change in electrolyte composition either on the outside or on the inside of the cell membrane. The composition of the outside was maintained constant and consequently the most likely change is a decrease in intracellular potassium with a concomitant increase in intracellular sodium. As pointed out earlier the permeability of the membrane for sodium is probably reduced by the detergents and therefore it seems unlikely that the change in reversal potential is caused by an increase of intracellular sodium (Na_i) due to an increase in the net sodium flux. A tentative explanation may be that the pumping mechanism in the membrane is inhibited and therefore the extrusion of sodium diminished. As a consequence of this the interior of the cell would gain sodium and loose potassium thereby changing the reversal potential for the SIC.

It is noteworthy that it thus would appear that Triton detergents mainly affect the properties of the outer membrane of the stretch receptor neuron. The impulse generation is considerably more sensitive to the action of Triton detergents than the transducer action while the spikes are blocked within 20-30 min the transducer functions of the neurons are not abolished until after 90 min. These effects are produced without significant changes of the resting membrane potential. The conclusion therefore is that Triton X-45 and Triton X 100 block not only the sodium channels involved in the generation of impulses but also the sodium current of the transducer membrane. The mechanism for this selectivity is unknown at present. The permeability for other ions involved in the production of the conducted and the non-conducted activity of the cell may also be affected.

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Capillary supply of the muscle fibre population in hindlimb muscles of the cat

By

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Abstract

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Comparative analyses of the fibre content (FG, FOG, and SO fibres) and the capillary density (the number of capillaries surrounding individual fibres and the capillary/fibre ratio) were performed in hind limb muscles of the cat. Cross-sections from the tenuissimus, the isopso femoris, the lateral head (LG) and the medial head (MG) of the gastrocnemius and the soleus are cut in cryostat. The sections were stained histochemically for the NADH₂-dehydrogenase and oxidase (pH 9.4) activities and ATPase activity which enables differentiation of different types of fibres. The endothelium of the capillaries was identified as staining for nonspecific alkaline ATPase activity. The number of capillaries surrounding each individual muscle fibre had a positive correlation, first to the oxidative capacity and secondly to the average diameter of the fibres. The thin tenuissimus muscle did not differ in this respect from the thicker muscles. The highest proportion of SO fibres was found in the soleus and the MG muscles. FG fibres of two different types were dominating the fibre mass in the isopso femoris and the LG muscles, while the tenuissimus contained more FOG fibres than these muscles. In general the FG fibres had a larger diameter than the FOG and the SO fibres. The soleus and the MG muscles contained larger fibres than the other examined muscles. FG fibres are surrounded by fewer capillaries than FOG and SO fibres. The soleus and the MG muscles, with higher percentages of SO fibres and also larger fibres, had the largest number of capillaries around the fibres and the highest capillary/fibre ratio.

Key words: Hind limb muscles, muscle fibre population, muscle fibre diameter, capillary density

The microvascular bed in the cat tenuissimus muscle has previously been analysed with intravital microscopy (Eriksson and Myrhaug 1972). Since this muscle is rather thin and small it is not unequivocally representing skeletal muscle tissue in wider aspects. The present study deals with comparative analyses of the muscle fibre population and the capillary supply in the tenuissimus and thicker hind limb muscles in cats.

Detailed knowledge of the fibre composition in various skeletal muscles has been provided by histochemical techniques (see review by Close 1972, Khan 1976). Quantitative biochemical analyses have also been performed on muscles with known fibre content (Dawson and Romstrand 1964, Peter *et al.* 1972).

Romanul (1965) found that the number of capillaries, surrounding each individual muscle fibre, was directly proportionate to the activity of oxidative enzymes in the fibres. Similar observations have been reported by Andersen (1975). Contradictory to this, Flyvbjerg and Groom (1975) stated that the diameter of the muscle fibre is the only factor determining the number of capillaries in muscle tissue. However this statement was not based on actual measurements of the muscle fibre diameter.

Ariano *et al* (1973) have described the percentage distribution of different muscle fibre types in various cat muscles. There is, however, no comparative study available concerning the capillary density in these muscles, even if quite a few authors give data on capillary density in muscles of different fibre composition (see e.g. Hudlická 1973).

The present study will discuss the relationship between fibre type, fibre dimension and capillarization of different fibres in some cat hind limb muscles. The different muscle fibre types will be described as FG (fast twitch - glycolytic), FOG (fast twitch - oxidative-glycolytic) and SO (slow twitch - oxidative). These terms were proposed by Peter *et al* (1972) and are corresponding to the FF, FR, and S fibres defined by Burke *et al* (1971). FG, FOG and SO are also corresponding to the terms A, B and C fibres, respectively, in previous publications (Eriksson and Myrhaug 1972, Myrhaug and Hudlická 1976).

Materials and Methods

8 adult cats of both sexes, weighing about 2.5 kg, were used. They were anesthetized with chloralose by injection of 50 mg/kg after induction with ether.

The examined muscles (the tenuissimus, the dorsal part of the biceps femoris, both heads of the gastrocnemius, and the soleus) were carefully exposed and transverse segments, 10 mm long, were excised from 3 different parts of each muscle (15 mm distal to the origin and 15 mm proximal to the insertion and one segment from the centre of the muscle belly). In the tenuissimus muscle, however, one segment was taken from the usual area of intra-fibre microscopy which is located about 10 mm distal to the nutritive artery emerging from the popliteal fat pad (cf. Myrhaug and Eriksson 1977). The other two segments were taken 20 mm proximal and 20 mm distal to this area.

The muscle segments were kept in ice-cold Histocon (Histo-Lab, Göteborg, Sweden) for up to 1 h, before freezing was performed in liquid propane-propane (Gärolf). After mounting on cryostat chucks the specimens were stored at -70°C . In a cryostat (-20°C), serial sections (7–10 μm thick) were cut perpendicularly to the muscle fibre direction. To enable qualitative fibre differentiation, the sections were stained histochemically for the activity of NADH₂-dehydrogenase (showing the oxidant capacity (Cheyette *et al* 1973)) and alkaline (pH 9.4) actomyosin ATPase (Padykula and Herman 1955). The method of unspecific alkaline ATPase described by Guth and Samaha (1969, 1970) was used for histochemical staining of capillary endothelium.

In 3 of the 8 animals the hind legs were fixed by 3% glutaraldehyde (buffered with 0.075 M sodium cacodylate to pH 7.2) via perfusion, at a pressure of 13.3 kPa (100 mmHg), through a cannula placed in abdominal aorta. To optimize the filling of the microvascular bed, vasodilatation was induced with papaverine (0.2 mg/kg) and preperfusion was undertaken with Perfadex[®] (cf. Myrhaug and Eriksson 1977). Muscle segments, as described above, were excised and embedded in Epon and serial sections (1 μm thick) were cut, perpendicular to the muscle fibres, in an ultratome. In sections, stained with toluidine blue, the capillaries could easily be identified (Eriksson and Myrhaug 1972).

For description of the fibre population in the examined muscles, 10 entire muscle fibre bundles (i.e. 300–400 fibres) were evaluated in each of the serial sectioned muscle segments. From each section a train of bundles, extending from the central area towards the periphery of the muscle belly, was photographed in a Leitz Orthomat automatic microscope camera (Leitz-Wetzlar, W. Germany). By comparing photographs from serial sections, alternately stained for dehydrogenase-actomyosin ATPase and unspecific alkaline ATPase activity, different fibre types could be distinguished and quantified. Fibre diameters were calculated

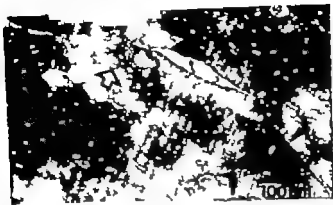


Fig. 1. Ultrathin section from the medial head of the gastrocnemius muscle (glutaraldehyde fixation). Small arrow indicates the boundary of muscle fibre bundle; open arrows show the capillaries.

By taking the mean of 4 polygonal diameter measurements, on each of the studied fibres, in calibrated photomicrographs (Cotter *et al.* 1973). The number of capillaries surrounding each muscle fibre was microscopically registered.

Both glutaraldehyde fixed sections and sections stained for unspecific alkaline ATPase activity were used for evaluation of capillary density. The numbers of capillaries per muscle fibre (the capillary/fibre ratio) and per cross sectioned area were calculated in 20 adjacent fibre bundles from each muscle segment.

Comments on methods

The muscle fibre bundle

Because of its fairly uniform fibre arrangement, the bundle (Fig. 1 and 2) was judged to be the most representative unit for descriptions of the fibre population. The bundle is enclosed by a delicate sheath of perimysium and contains about 20–80 muscle fibres, each are surrounded by a thin layer of endomysium (Myrberg and Eriksson 1977). The entire fibre mass was grouped into fibre bundles in all the examined muscles. In the trapezius, biceps femoris and gastrocnemius muscles most of the large fibres, with low oxidative capacity, were located close to the periphery of the bundles, while the smaller fibres with higher oxidative capacity were situated deeper in the bundles.

Capillary visualization

In muscle sections stained *ad sensum* Padykula and Herman (1955), the alkaline ATPase activity in the different muscle fibres reacted quite intensively. The capillaries are, however, only sparsely and unevenly revealed. In the sections stained for unspecific alkaline ATPase activity (Guth and Saxena 1970) the capillaries are distinctly delineated (Fig. 2d). Capillary endothelium has been shown to contain high activity of alkaline phosphatase (Gomori 1939). ATP (which is used as substrate in methods for ATPase) can be hydrolysed by alkaline phosphatase as well as by different specific ATPases (Padykula and Herman 1955, Frome and Kaplan 1960). Since the method by Guth and Saxena (1970) contains no specific inhibitor for alkaline phosphatase, seems to be quite comparable with the method of Gomori (1939) and all capillaries present in the sections, should be stained by either of these two methods.

The capillaries can also be identified in muscle sections fixed by glutaraldehyde (Fig. 1; see also Eriksson and Myrberg 1972).

Results

Muscle fibre orientation

The muscle fibres in the trapezius muscle ran parallel to the longitudinal axis of the muscle. A similar relationship existed in the dorsal part of the biceps femoris and in the

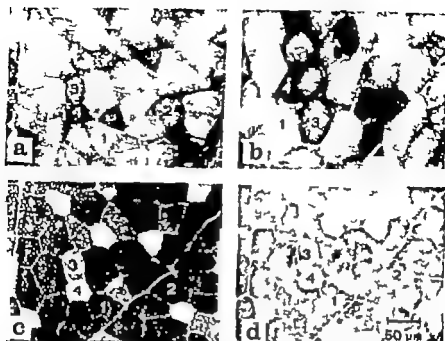


Fig. 2. Cryostat sections from the lateral head (a, c and d) and the medial head (b) of the gastrocnemius muscle. Histochemical staining for NADH₂-diaphorase activity (a, b), alkaline ATPase activity *ad modum* Padykula and Herman (c) and *ad modum* Guth and Samaha (d). Fibre types: FG (1), FG/FOG (2), FOG (3) and SO (4).

lateral head of the gastrocnemius (LG) In the medial head of the gastrocnemius (MG) and in the soleus muscle the fibres were, however, oriented obliquely to the longitudinal axis of the muscle (Myrhaage and Eriksson 1977)

Fibre populations

Different fibre types could be distinguished by their staining intensity for alkaline ATPase- and NADH₂-diaphorase-activity (denoted here as ATPase activity/diaphorase activity): FG - high/low FOG - moderate/high close to the sarcolemma but low to moderate in the centre of the fibre, FG/FOG - high/as in FOG fibres, SO - low/moderate to high, but evenly distributed (Fig. 2)

FG and FOG fibres, taken together were most common in the tenuissimus, the biceps femoris and the LG muscles, where such fibres constituted about 85 % of the total fibre population. More than half of this amount consisted of FOG fibres in all examined ten-

TABLE I. Muscle fibre populations (mean \pm S.E. $n = 4$).

Fibre types (%)	Tenuissimus	Biceps femoris	Gastrocnemius		Soleus
			LG	MG	
FG	35 \pm 1.1	41 \pm 2.3	50 \pm 0.8	4. \pm 1.1	—
FG/FOG	—	12 \pm 1.1	11 \pm 0.9	—	—
FOG	49 \pm 2.1	33 \pm 2.1	24 \pm 0.9	22 \pm 1.8	1 \pm 0.4
SO	16 \pm 1.1	14 \pm 2.0	15 \pm 0.6	36 \pm 1.3	99 \pm 0.4

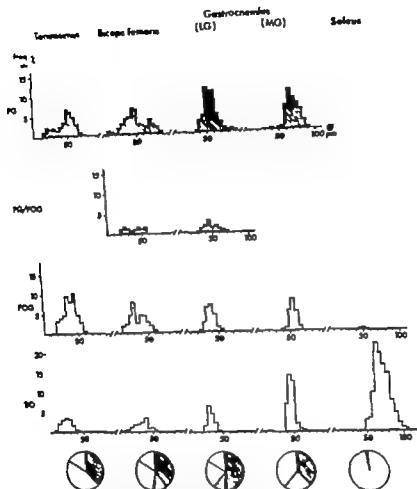


Fig. 3. Frequency distribution of fibres of different type and diameter (percentage values from one animal). The total percentage of each fibre type is illustrated as sectors in the circles.

some muscles, while the FG fibres were dominating in the biceps femoris and the LG muscles. FG/FOG fibres were found, as a regular fibre type only in the LG and the biceps muscles, where they formed 11 to 12% of the total fibre population (Table I). The highest content of SO fibres was found in the soleus muscles (on average $99 \pm 0.4\%$ /mean \pm S.E.,

4) and in the MG muscle (on average $36 \pm 1.3\%$).

Fibre dimensions

The FG fibres had the largest average diameter of the fibre population, in all evaluated segments from the tenuissimus, biceps, LG and MG muscles (Fig. 3 and Table II).

The MG muscles contained the largest FG fibres (average diameter $69 \pm 0.7 \mu\text{m}$) in all the animals. These fibres were considerably larger than in the LG muscles (on an average:

TABLE II Average diameters of muscle fibres (μm mean \pm S.E.; $n=4$).

Fibre types	Tenuissimus	Biceps femoris	Gastrocnemius		Soleus
			LG	MG	
FG	47 ± 2.4	50 ± 2.6	57 ± 1.8	69 ± 0.7	—
FG/FOG	—	37 ± 2.5	48 ± 1.7	—	—
FOG	38 ± 1.8	35 ± 1.7	39 ± 2.0	55 ± 0.6	51 ± 1.0
SO	25 ± 1.7	32 ± 2.9	33 ± 1.4	47 ± 0.4	72 ± 1.9

$57 \pm 1.8 \mu\text{m}$) In the tenuissimus and the biceps muscles, the dimensions of the FG fibres were smaller and not different from each other (average values $47 \pm 2.4 \mu\text{m}$ and $50 \pm 2.6 \mu\text{m}$, respectively).

The range of the FG fibre diameters was widest in the biceps femoris and the tenuissimus muscles, in all animals, as shown in Fig. 3.

The FOG fibres had a similar average diameter in the tenuissimus, biceps and the LG muscles (average values 38 ± 1.8 , 35 ± 1.7 and $39 \pm 2.0 \mu\text{m}$, respectively). In the MG muscles they were, however considerably larger ($55 \pm 0.6 \mu\text{m}$). In the soleus muscles there were only quite few FOG fibres (always less than 3% of the evaluated fibre mass) and these fibres had an average diameter comparable to the same type of fibre in the MG muscles (Table II).

The transitional group of fibres (FG/FOG) with a high activity of alkaline ATPase but with an oxidative capacity similar to the FOG fibres, also had an average diameter similar to FOG fibres in the biceps muscles. In the LG muscles the diameters of the FG/FOG fibres were ranging in between the values of FG and FOG fibres (see Table II).

The SO fibres were considerably larger in the MG muscles ($47 \pm 0.4 \mu\text{m}$) as compared to the tenuissimus, biceps and LG muscles (25 ± 1.7 , 32 ± 2.9 and $33 \pm 1.4 \mu\text{m}$, respectively). In the tenuissimus and the biceps muscles quite small SO fibres (about 10–15 μm in average diameter) were found to a varying extent in all segments from these muscles, as shown in Fig. 3.

The soleus muscles were almost entirely (97–100%) composed of SO fibres with an average diameter of $72 \pm 1.9 \mu\text{m}$.

Number of capillaries around fibres

The absolute values for the number of capillaries around different muscle fibres varied from 0–5 in the tenuissimus, biceps and the LG muscles, from 0–7 in the MG muscles and from 2–9 in the soleus muscles.

In spite of its larger fibre diameter the FG fibres, in all examined tenuissimus, biceps, LG and MG muscles, had a smaller number of surrounding capillaries (range 1.5–3.3) than the FOG fibres (range 2.0–3.8). The few FOG fibres found in the soleus muscles, had an even higher number of surrounding capillaries (on an average 4.8). For the FG/FOG fibres the number of fibre surrounding capillaries were in the range of the figures for the FG and the FOG fibres (Fig. 4 and 5).

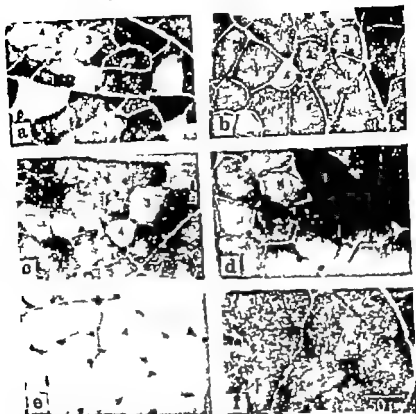


Fig. 4. Capillary density in cryostat sections from the tenosusmus (e), the dorsal part of the biceps femoris (b), the lateral head (b) and the medial head (c) of the gastrocnemius and the soleus (d). All these sections are stained for aspecific alkaline ATPase of medium Oxid and Saponin. The even distribution of formazan granules in μ sections from the soleus (stained for NADH₂-dehydrogenase activity) is shown in (f). Fibre types: 1) FG 2) FG/FOG 3) FOG 4) SO

The SO fibres in the soleus muscles had a similar number of surrounding capillaries as the FOG fibres (average values 5.0 and 4.8, respectively). In the other 4 muscles the SO fibres had a number of surrounding capillaries (range: 2.0–3.4) which was slightly lower or similar to the corresponding values for the FOG fibres (Table III).

As summarized in Fig. 5 a, the biceps femoris and the LG muscles had fewer capillaries around their fibres as compared to the MG and the soleus muscles, where also all types of fibres had larger average diameters. Furthermore, the FG (and FG/FOG) fibres were dominating in the biceps and the LG muscles, while the MG and the soleus muscles had more of SO fibres. The tenosusmus muscles had a higher content of FOG fibres and these muscles fell in between the biceps-LG and the MG-soleus groups regarding the number of fibres surrounding capillaries.

The fibre diameter and the number of surrounding capillaries in different muscles had a positive correlation (Fig. 5 b), however the FG and the FG/FOG fibres had notably lower values for the number of capillaries around fibres.

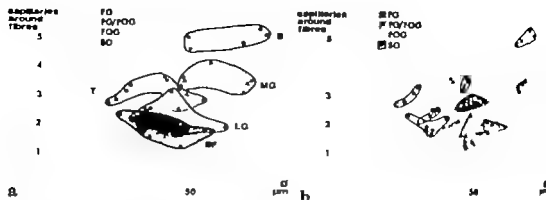


Fig. 5 The average number of capillaries around individual muscle fibres, in relation to fibre type and average diameter of the fibres (in four animals). a) values grouped for different muscles, b) values grouped for different fibre types. Te = tenuissimus, BF = biceps femoris, LG = the lateral head and MG = the medial head of the gastrocnemius, S = the soleus.

Capillary density

The capillary/muscle fibre ratio was calculated from examinations of 20 adjacent muscle fibre bundles in each muscle segment and the average values for the animals ranged from 0.78–1.92 in sections stained for unspecific alkaline ATPase and from 0.76–1.87 in the sections from glutaraldehyde perfused muscles.

The biceps femoris and LG muscles had the lowest values (range in 7 animals 0.74–0.84) and the MG and soleus muscles had the highest values (range 1.04–1.17 and 1.83–1.94 respectively). The tenuissimus muscles showed intermediate values, ranging from 0.89 to 1.02.

The capillary density per mm² of cross section area was 657 ± 54 (mean \pm S.E., $n=4$) for the tenuissimus muscle. The corresponding values for the biceps femoris, the LG, the MG and the soleus muscles were 617 ± 66 , 570 ± 34 , 695 ± 49 and 948 ± 47 respectively.

In general the values from the glutaraldehyde perfused muscles were only slightly smaller than the corresponding values from the muscles stained for activity of unspecific alkaline ATPase (Table III).

TABLE III Average number of capillaries around muscle fibres and capillary/fibre ratio (mean \pm S.E.)

Fibre types	Tenuissimus	Biceps femoris	Gastrocnemius		Soleus
			LG	MG	
<i>Capillaries around muscle fibres ()</i>					
FG	4.7±0.11	1.5±0.11	1.9±0.09	3.3±0.09	—
FG/FOG	—	1.8±0.13	1.9±0.15	—	—
FOG	1.4±0.09	2.0±0.06	2.8±0.13	3.8±0.13	4.8±0.10
SO	4.9±0.09	2.0±0.14	2.3±0.09	3.4±0.09	5.0±0.11
<i>Capillary/fibre ratio</i>					
(*)	0.95±0.03	0.78±0.09	0.83±0.08	1.13±0.02	1.92±0.01
()	0.92±0.02	0.79±0.02	0.76±0.01	1.10±0.04	1.87±0.02

() from sections stained for ATPase ($n=4$).

(*) from glutaraldehyde perfused muscles ($n=3$).

Discussion

The present results indicate that the amount of capillaries in skeletal muscle tissue has a positive correlation firstly to the oxidative capacity of different muscle fibres, and secondly the dimension of the fibres.

Muscle fibre populations

The medial head of the gastrocnemius (MG) was found to contain more slow twitch-oxidative (SO) fibres than the lateral head (LG) of the same muscle. The LG muscle had essentially the same fibre population as the biceps femoris muscle. This is in accordance with the findings by Ariano *et al.* (1973).

The content of fast twitch-oxidative-glycolytic (FOG) fibres in the tenuissimus (and also in the biceps, LG and MG muscles) was higher in the present study as compared to figures presented by Ariano. The discrepancy might be due to a higher level of exercise in the cats used in the present study since 75% of them had been confined to cages for only 3-4 weeks before the expts. It has been shown that exercise increases the activity of oxidative enzymes in muscle (*cf* Holloszy *et al.* 1971) and also the number of muscle fibres corresponding to the FOG type (Barnard *et al.* 1970, Jamson and Kaijer 1977).

Almost all fibres in the soleus muscles from the present expts. had an even distribution of formazan granules (NADH₂-diaphorase reactions) which is typical for SO fibres. This finding corresponds well with observations by several authors (*e.g.* Tsukamoto and Mori 1966, Ariano *et al.* 1973, Hammarberg 1974).

A fraction of fast twitch-glycolytic (FG) fibres, with a notably high activity of NADH₂-diaphorase (similar to the one found in FOG fibres) was found in the biceps and the LG muscles. This type of fibre was also observed by Hammarberg (1974) in the cat LG muscle.

Muscle fibre diameters

The degree of muscle tension would not affect the fibres diameter values, since fibre perpendicular *cross-sections* of the examined muscles were frozen instead of whole muscles.

The FG fibre was the largest fibre type in the tenuissimus, biceps femoris, LG and MG muscles. The average diameter of the FOG and SO fibres was about 20-25% smaller than that of the FG fibres, and the SO fibres were generally slightly smaller than the FOG fibres. All 3 fibre types in the MG muscles were considerably larger than corresponding fibres in the tenuissimus, biceps and LG muscles. The soleus muscle contained SO fibres with an average diameter about 50% larger than the SO fibres in the MG muscles and more than 100% larger as compared to the corresponding fibres in the three other muscles.

The fibre dimensions in the tenuissimus and the MG muscles are quite similar to the data presented by Boyd (1956) and Henneman and Olson (1965). In the present study the 4 different fibre types of the LG muscle are all smaller than the corresponding figures reported by Hammarberg (1974). The method for estimation of fibre diameters used by Hammarberg was 'the mean of the two largest diameters' while the present estimations were based on 4 polygonal diameters. This would reasonably explain the difference in diameter values in

these two studies. Furthermore, entire, adjacent muscle fibre bundles were examined in the present study instead of "randomly chosen areas" as in the study by Hammarberg.

The average fibre diameter for the soleus muscles, in the present study is similar to the values found by Hammarberg, but about 15% larger than the figure presented by Henneman and Olson (1965).

The frequency distribution for the fibre diameters had a wide range in the tenuissimus, biceps and LG muscles, which all have fibres oriented parallel to the longitudinal axis of the muscle belly. Especially the tenuissimus and the biceps muscles contained several quite small fibres (10–15 μm in diameter). For the tenuissimus muscle, this could be explained by the existence of interdigitating, only 2 cm long muscle fibres as described by Adrian (1925).

Capillary supply of muscle fibres

The larger fibres in the MG and soleus muscles were surrounded by more capillaries than the fibres in the biceps femoris and LG muscles. In general, the FOG and the SO fibres had slightly higher values of surrounding capillaries than the FG and the FG/FOG fibres.

Since the diameters of the fibres in the tenuissimus were not exceeding the diameter of fibres in the biceps and LG the numbers of fibre surrounding capillaries in the tenuissimus muscle were comparably large. This might be explained by the high proportion of FOG fibres found in the tenuissimus muscle. Several FG fibres would accordingly share their capillaries with bordering FOG fibres.

The high number of fibre surrounding capillaries found in the soleus and the MG muscles as well as the lower values for the biceps and LG and the medium values for the tenuissimus were confirmed by the calculated figures of capillary/muscle fibre ratio. The sections stained for unspecific alkaline ATPase and the sections from the glutaraldehyde perfused muscles had approximately the same capillary density.

The number of capillaries around each fibre increased parallel with increasing muscle fibre diameter. However the FOG and SO fibres were surrounded by more capillaries than the comparatively larger FG fibres, which means that muscle fibres with high oxidative metabolism were found to be supplied by more capillaries than fibres with low oxidative metabolism.

This is in accordance with findings presented by e.g. Ranvier (1874), Stool (1925), Lee (1958) and Romanul (1965). Similar findings were also reported by Andersen (1975), while Plyley and Groom (1975) stated that, the muscle fibre diameter was the only factor determining the number of capillaries surrounding the fibre.

A direct relationship between oxidative capacity of muscle fibres and the capillary density in the muscle has also been indicated by physiological experiments. Hilton (1968, Hilton *et al* 1970) observed that a "red" muscle, with high oxygen consumption (cat soleus) had a maximal blood flow capacity which was 2–3 times higher than that of the "white" gastrocnemius muscle. This finding was confirmed by Folkow and Halicka (1968) and by Reis and Wooten (1970).

Furthermore, chronic stimulation of rabbit fast muscles at a low impulse frequency (10 Hz) has been shown to increase the activity of oxidative enzymes but decrease the average diameter of the muscle fibres. Still the capillary/fibre ratio increased by 20% after only

days and by 55% after 28 days of stimulation (Cotter *et al.* 1973, Pette *et al.* 1973, Brown *et al.* 1976, cf. Myrhaug and Hudlicki, 1977).

Thus, the activity of oxidative enzymes in the individual muscle fibres seems to be, even more important than the fibre diameter for the capillary density and reasonably also for the number of capillaries surrounding each fibre in skeletal muscle tissue. The present study shows that the tenuissimus muscle in cat is not different from thicker hindlimb muscles in this respect.

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Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22-65 years

By

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Abstract

LARSSON, L., B. SÄÖREN and J. KARLSSON. *Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22-65 years.* Acta physiol. scand. 1978, 103, 31-39.

Biopsies for histochemical and biochemical analysis were taken from the vastus lateralis muscle of 35 sedentary, healthy male subjects from III to 65 years of age. Fibre type distribution changed towards I fibres with increasing age. The percentage of type II fibres, both in type IIA and type IIB fibres, decreased with age. The percentage of type IIC fibres did not change with increasing age. It was found that the type IIB/IIA fibre ratio was inversely related to type I fibres, i.e. subjects rich in type I fibres had relatively smaller proportion of type IIB fibres. Fibre area determinations revealed selective decrease in type II fibre area. Consequently the type I/II fibre area ratio and relative type II fibre area decreased. No changes in the specific activities of Mg^{++} stimulated ATPase and myokinase were observed, while the activity of lactate dehydrogenase (LDH) was higher in the youngest groups than in the oldest. LDH isoenzyme pattern shifted towards decrease in percentage distribution of the muscle specific isoenzymes and corresponding decrease in muscle specific activity. In the activity of the heart specific isoenzymes did not change.

During the main part of man's productive period of life, ageing processes take place to varying degrees in all organ systems. Perhaps some of the most prominent changes are the decrease in muscular strength and decline in the fine co-ordination of movements (Ulfand 1933, Barren 1952, Burke *et al.* 1953, Astrummen and Hochell-Nielsen 1961). It is reasonable to assume that one important part of the ageing process causing these deficiencies takes place in muscle tissue itself. The objective of the present study has been to investigate the histochemical and some of the biochemical characteristics of importance for muscle contractility and endurance of a relatively homogeneous male population in terms of occupational and spare time physical activity in the age range of 22 to 65 years.

Material and methods

35 healthy male volunteers (22-65 yrs) participated in the study. They were divided into different age groups, 20-29, 30-39, 40-49, 50-59 and 60-65 years. The number of subjects per age group varied from

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TABLE I. Fibre type distributions, fibre areas, fibre area ratios and relative fibre area of type II fibres in the muscle of the different age groups studied.

Age group	Mean age (yr)	Number	% Type I fibres	Area μm^2		Area ratio Type II/I	% Area Type II fibres
				Type I	Type II		
20-29	26	11	40.5 \pm 3.9 (14-59)	5 666 \pm 499 (2 851-8 704)	6 933 \pm 588 (4 562-9 507)	1.24 \pm 0.05 (1.04-1.60)	63.7 \pm 4.3 (43.1-89.2)
30-39	36	12	36.5 \pm 1.6 (27-44)	6 344 \pm 397 (3 963-8 931)	6 975 \pm 316 (4 872-9 981)	1.17 \pm 0.13 (0.69-2.05)	67.0 \pm 2.1 (55.5-72.9)
40-49	43	10	48.1 \pm 4.3 (29-67)	6 754 \pm 398 (4 939-8 670)	6 627 \pm 340 (4 224-9 558)	0.96 \pm 0.06 (0.84-1.29)	50.9 \pm 3.8 (31.8-63.9)
50-59	53	12	51.7 \pm 3.0 (28-68)	5 941 \pm 627 (2 663-9 005)	5 954 \pm 449 (4 072-9 003)	1.04 \pm 0.09 (0.81-1.69)	49.3 \pm 4.5 (37.2-72.0)
60-65	61	10	55.0 \pm 4.3 (38-79)	5 991 \pm 343 (4 120-7 275)	5 243 \pm 203 (4 230-6 457)	0.96 \pm 0.05 (0.74-1.18)	43.6 \pm 4.3 (18.8-65.4)

Values are means \pm S.E. Values in parentheses are highest and lowest observations.

fibre area ratio decreased ($p < 0.05$) with age (Fig. 2). Consequently the relative type II fibre area decreased ($p < 0.001$) from 64% to 44% in the 20-29 and 60-65 year old groups, respectively.

No significant change in the enzyme activity of Mg²⁺ stimulated ATPase or myokinase (Mk) with age was observed. However lactate dehydrogenase (LDH) activity was found to be significantly ($p < 0.05$) higher in the younger groups as compared with the older. LDH enzyme pattern shifted towards less muscle-specific isozymes with age as indicated by the percentage distribution M/LDH ($p < 0.01$) (Table III). This took place by means of a significant ($p < 0.01$) decrease in the activity of muscle-specific isozymes with age. The activity of heart-specific isozymes tended to increase although not significantly (Fig. 3). Consequently the M/H activity ratio decreased ($p < 0.05$).

TABLE II. Fibre type distribution as type I, IIA, IIB and IIC in 41 of the 55 subjects in Table I.

Group	Mean age (yr)	Number	% Type I fibres	Type IIA fibres	Type IIB fibres	Type IIC fibres
20-29	26	7	39.1 \pm 3.4 (15-56)	34.0 \pm 2.8 (27-41)	24.1 \pm 4.1 (15-43)	2.7 \pm 0.6 (1-5)
30-39	36	11	36.1 \pm 1.6 (28-44)	38.6 \pm 2.4 (26-51)	22.1 \pm 2.1 (14-34)	4.0 \pm 1.2 (1-11)
40-49	43	7	49.6 \pm 5.2 (37-75)	33.6 \pm 3.1 (22-47)	15.0 \pm 4.5 (2-28)	1.9 \pm 0.9 (0-7)
50-59	53	9	49.4 \pm 5.1 (23-72)	28.7 \pm 2.7 (14-40)	19.4 \pm 3.9 (4-37)	1.6 \pm 0.6 (0-5)
60-65	62	7	65.6 \pm 4.8 (46-85)	19.6 \pm 2.1 (13-29)	12.9 \pm 4.3 (1-36)	2.3 \pm 0.6 (1-4)

Values are means \pm S.E. Values in parentheses are highest and lowest observations.

10-12. The subjects were employees of an insurance company (clerks) and were untrained to moderate trained (group I-II) according to Salin and Grimby (1968) in respect to occupational and spare time physical activity).

Muscle biopsies were obtained from the left vastus lateralis muscle using the needle biopsy technique (Bergström 1962). Biopsies for enzyme activity analyses were immediately frozen in liquid nitrogen and stored at -80°C . Enzyme activity was determined by fluorometric means using NAD-NADP coupled reactions according to Lowry and Passonneau (1972). The enzymes investigated were Mg^{2+} stimulated ATPase (E.C. 3.6.1.4), myokinase (MK) (E.C. 2.7.4.3) and lactate dehydrogenase (LDH) (E.C. 1.1.1.27). The LDH isozymes in muscle samples were separated by means of disc-electrophoresis and stained. The intensity of stained bands corresponding to the LDH isozymes was quantified by means of densitometric scanning according to Penney *et al.* (1974). The relative quantity of M and H subunits was calculated according to Thorling and Jensen (1966). Biopsies for histochemical analyses were trimmed, mounted frozen in isopentane cooled with liquid nitrogen and stored at -80°C until analysis.

Serial transverse sections (10 μm) were cut with a microtome at -20°C and stained for myofibrillar ATPase (Gomori 1941; Padykula and Herman 1953) and for NADH-diaphorase activity (Novikoff *et al.* 1961). Photographs of the stained sections were taken and fibre classification into type I and II (Engel 1962) was made in all 55 subjects. In 41 of the subjects the type II fibres were subclassified into A, B and C subgroups (Brooke and Kaiser 1970; Dubowitz and Brooke 1973). No significant difference in percentage distribution of type I and type II fibres was found between the groups consisting of 41 and 55 subjects, respectively. The mean number of counted fibres for classification into main groups (type I and type II) and subgroups (type I type IIA, B and C) was 602 ± 52 and 265 ± 32 , respectively. Fibre area determination was made from the photograph of the NADH-diaphorase staining via a cutting and weighing technique (for detailed information see Thorstensson *et al.* 1977).

Ordinary statistical methods were used to calculate means and standard error of the means (S.E.). The significance of linear correlation coefficients was tested from individual values according to Siedecor and Cochran (1967).

Results

The average muscle fibre type distribution, fibre area and relative fibre area occupied by each fibre type in the muscle are shown in Table I and Table II. Fibre type distribution data for all 55 subjects displayed a significant ($p < 0.001$) linear increase in the percentage of type I fibres with increasing age (Fig. 1). Thus, the average type I fibre distribution was 55% in the 60-65 year old group compared with 41% in the 20-29 year old group. Type II fibre subclassification revealed no significant change in type IIC fibre distribution (range 2-4%), whereas the percentile distribution of type IIA and type IIB both decreased significantly ($p < 0.001$ and $p < 0.05$ respectively). Type IIA averaged 34% in the 20-29 year old group as compared to 20% in the 60-65 year old group. The type IIB/IIA fibre ratio did not, on the other hand, change with age, although a tendency to an increase was observed. Neither could any significant difference be observed between means of the two youngest groups as compared with means of the two oldest groups.

When the individual data were taken together type IIB/IIA fibre ratio demonstrated an inverse relationship versus type I fibre distribution ($r = 0.55$, $p < 0.001$). This pattern was also true for all age groups studied although significant only for some groups. This means that a subject irrespective of age with a high percent of type I fibre in his skeletal muscle, simultaneously had a relatively lower proportion of type IIB fibres.

The fibre area determinations revealed a selective linear decrease in type II fibre area ($p < 0.01$), while no significant change in type I fibre area could be seen. The type II fibre area decreased from 6853 μm^2 in the 20-29 year old group to 5243 μm^2 in the 60-65 year old group. This corresponds to a 33% decrease in absolute type II fibre area. The type II/I

TABLE III. Enzyme activities of Mg²⁺ ionized ATPase, myokinase and total dehydrogenase (LDH). Lactate dehydrogenase activity is distributed as follows: LDH1 and M-LDH1 and M-LDH2 and as M-LDH1% of LDH total.

Group	Mean age (yr)	Number	Mg ²⁺ ATPase 10 ⁻⁴ moles mg protein ⁻¹ min ⁻¹	Myokinase 10 ⁻⁴ moles mg protein ⁻¹ min ⁻¹	Lactate dehydrogenase (LDH) 10 ⁻⁴ moles g ⁻¹ min ⁻¹	PA → LA	LA → PA	H-LDH	M-LDH	% M-LDH of total LDH
20-29	26	11	11.7 ± 1.3 (6.9-20.0)	146.4 ± 7.3 (108.2-179.0)	0.43 ± 0.06 (0.24-0.87)	0.31 ± 0.04 (0.22-0.64)	0.16 ± 0.02 (0.03-0.39)	0.32 ± 0.03 (0.16-0.65)	74.0 ± 3.1 (60-84)	
30-39	34	~12	10.8 ± 1.3 (6.4-19.5)	152.9 ± 12.1 (110.9-239.4)	0.39 ± 0.07 (0.16-0.95)	0.44 ± 0.07 (0.23-0.93)	0.15 ± 0.03 (0.04-0.45)	0.46 ± 0.06 (0.06-0.69)	70.9 ± 4.7 (45-91)	
40-49	45	10	11.5 ± 1.3 (5.3-18.8)	133.3 ± 13.0 (89.3-192.3)	0.42 ± 0.06 (0.11-0.78)	0.30 ± 0.07 (0.15-0.82)	0.16 ± 0.02 (0.08-0.29)	0.26 ± 0.03 (0.03-0.49)	65.4 ± 2.4 (54-77)	
50-59	55	12	9.9 ± 0.7 (5.6-13.1)	141.1 ± 13.3 (40.3-253.9)	0.48 ± 0.09 (0.17-0.90)	0.47 ± 0.05 (0.11-0.81)	0.16 ± 0.03 (0.04-0.54)	0.27 ± 0.07 (0.08-0.78)	62.7 ± 3.0 (44-88)	
60-69	62	~10	12.2 ± 1.0 (8.7-18.3)	142.1 ± 5.8 (114.7-171.4)	0.38 ± 0.09 (0.20-0.77)	0.44 ± 0.06 (0.29-0.64)	0.16 ± 0.03 (0.07-0.37)	0.23 ± 0.05 (0.13-0.41)	59.1 ± 3.3 (45-67)	

Values are means ± S.E. Values in parentheses are highest and lowest observations.

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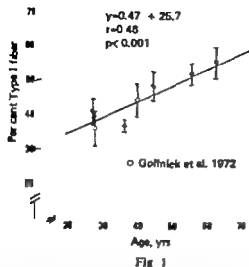


Fig. 1

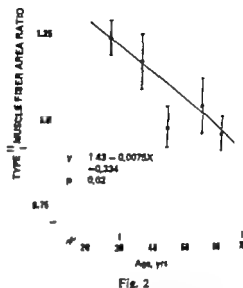


Fig. 2

Fig. 1 Percentage distribution of type I fibres (vastus lateralis muscle) versus age. Mean values \pm S.E. are shown along with the regression line. Regression line is calculated from individual values. Data from Golfinick *et al.* (1972) are also plotted.

Fig. 2 Type II fibre area ratio versus age. Mean values \pm S.E. are shown along with the regression line. Regression line is calculated from individual values.

Discussion

The main finding in the present study was the shift in fibre type distribution towards a higher percentage in type I fibres and a corresponding decrease in type II fibres with age. The percentage distribution of type IIA and type IIB fibres also decreased, while type IIB/TIA fibre ratio and type IIC fibres did not change significantly. Moreover, the type IIB/TIA fibre ratio was found to be inversely related to percentage type I fibres. A selective decrease in type II fibre area was found with age, while type I fibre area did not change significantly. The enzyme activity of Mg^{++} stimulated ATPase and myokinase remained unchanged, while the activity of lactate dehydrogenase (LDH) was found to be higher in the youngest groups as compared with the oldest. LDH isozyme pattern shifted towards a decrease in percentage distribution of the muscle-specific isozymes and a corresponding decrease in activity while the activity of the heart-specific isozymes did not change.

The subjects in the present study were recruited on the basis of an equal occupational and spare time physical activity level (Saltin and Grimby 1968) to get a population as homogeneous as possible. However, it cannot be ruled out that the subjects adapted to a more sedentary way of living with age, although having almost equal occupational and spare time physical activity levels. Irrespective whether this is an endogenous or a sociological-anthropological process, it must be regarded as a part of the overall ageing process with its significance for muscle tissue adaptation.

No systematical studies in healthy men at age 22 to 65 years are available. Golfinick *et al.*

anterior muscle of the rat, Tauchi *et al* (1971) have claimed that there is a decrease in the number and volume of type II fibres and in the number of type I fibres. This selective atrophy of type II fibres is in accordance with findings in the old rat soleus muscle (Bass *et al* 1973).

According to the animal studies quoted above, it seems likely that there is an increase in type II fibre area and a transformation of type II fibres into type I fibres during maturation, while in the ageing muscle there is a decrease in type II fibre area and a loss of muscle fibres. Whether the change in fibre type distribution taking place in the ageing muscle in the present study is due to a transformation of type II fibres cannot be answered, since the total number of muscle fibres have not been determined. The selective type II fibre atrophy seen in the ageing muscle also occurs in conditions such as denervation, inactivity and malnutrition (Engel 1965 Engel 1970).

Findings by Gutmann and Hanzlíková (1966) have shown that the metabolic profile of the muscles of an old animal resembles denervated muscles. The preferential atrophy of type II fibres in denervated muscles (Engel 1962) has been suggested to be due to a preferential susceptibility of type II fibres to loss of trophic influence from both type I and type II neurons (Karpau and Engel 1968, Engel 1970, Engel and Warmoths 1971). However human muscle fibre types differ in many respects from those of cat and rat (Schmalbruch and Karnesøen 1974). This must be taken into account when comparing the information available from animal and human studies. As has been pointed out above, it cannot be ruled out that the activity level decreases with age although the subjects have an almost equal occupational and spare time physical activity. This might cause the selective type II fibre atrophy. However at the present time it is impossible to evaluate whether the type II fibre atrophy is related to denervation/inactivity or both.

The changes found in LDH activity isoenzyme pattern and isoenzyme activity are in accordance with findings in fibre type distribution observed in the present study (Karlsson *et al* 1974). However it cannot be established whether the shift in isoenzyme activity is due to a transformation of type II fibres into type I fibres or a degeneration of type II fibres, since the activity in the whole muscle has not been measured.

The metabolic changes found in the ageing human muscle, *i.e.* an increasing percentage of type I fibres, a selective type II fibre atrophy and a decrease in the activity of the muscle specific LDH isoenzymes, might explain some of the changes found in physical capacity with age, such as increasing slowness of movement and decreasing muscular strength. Which of the underlying mechanisms may be causing these changes can only be speculated upon. According to data available so far from human and animal studies, the most probable cause for these metabolic changes is an atrophy and degeneration of the type II fibres rather than a transformation of the type II fibres. This degeneration of the type II fibres might be due to decreasing trophic influence from the motoneurons caused by denervation-like process.

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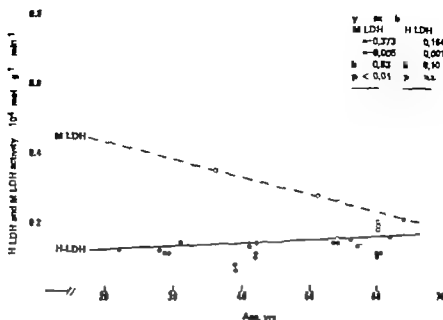


Fig. 3 M LDH and H LDH activity versus age, respectively. Individual values are shown along with regression lines.

(1972) compared muscle fibre type distribution in physically untrained and trained men. Their study provided data on the fibre type distribution in untrained men in two age groups, 24–30 and 31–52 years. The percentage of type I fibres amounted to 36% and 44%, respectively. The present data are in good agreement with Gollnick *et al.* 1972 (Fig. 1).

A recently published study (Tomonaga 1977), using histochemical and electron microscopic methods studied skeletal muscles in 79 elderly patients (mainly in the 60–90 age group). The most prominent findings in the senile muscle were the neuropathic changes (group atrophy, small dark angulated fibres, target fibres, type grouping and nuclear clumps) and type II fibre atrophy. It was also found that type II fibre atrophy was more common in the proximal muscles of the lower extremity than neuropathic changes, whereas the reverse relationship was found in the distal muscles of the lower extremity. Type II fibre area decreased ($p < 0.05$) with age while type I fibre area remained unchanged. Consequently the type II/I fibre area ratio decreased ($p < 0.01$), a circumstance in agreement with findings in the present study.

Studies on rat soleus muscle during growth (Kugelberg 1976) have shown an increasing percentage of type I fibres with age and a corresponding decrease in the percentage of type II fibres. Similar data were obtained by Karpati and Engel (1967) during maturation in guinea pig, rat and cat soleus muscle. The latter authors suggested this to be due to a transformation of type II fibres into type I rather than atrophy or degeneration of the type II fibres, since the total number of fibres increased and the diameter of the type II fibre gradually increased until they disappeared. According to Kugelberg, the transformation is due to changing properties of the motoneurons, *i.e.* from phasic to tonic. Other studies (Gutmann and Hanzlíková 1966, Bass *et al.* 1975) examining the old rat soleus muscle have reported a loss of muscle fibres and a decrease in fibre size in the soleus muscle. On the basis of percentage changes in type I fibres and decrease in number of fibres in the senescent

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enzymes, i.e. phosphofructokinase (E.C.2.7.1.11.), glycogen phosphorylase (E.C.2.4.1.1.) and lactate dehydrogenase ((LDH) E.C.1.1.1.27), in the two main muscle fibre types (Essén *et al.* 1975, Sjodin 1976, Thorstensson 1976). Thus, both LDH activity and the LDH isozyme distribution pattern in an untrained or moderately trained human population were found to be related to the percentage of FT fibres in the muscle (Karlsson *et al.* 1974). A muscle rich in FT fibres displayed greater LDH activity and a larger contribution of muscle-specific LDH (M-LDH) than a muscle rich in ST fibres in which the heart-specific type of LDH (H-LDH) is predominant. The intracellular distribution of these two main LDH isozymes and their kinetic properties differ (for ref. see Sjodin 1976). In normal conditions, the M-LDH isozymes are mainly associated with membranes, such as the sarcoplasmic reticulum. On the other hand, H-LDH fractions have been found in the inner membrane of the mitochondria.

The aim of the present investigation was to study lactate accumulation in the muscles of individuals differing with respect to certain relevant muscle qualities. Therefore, muscle lactate concentration was determined after intense, standardized exercise of brief duration and related to individual muscle fibre type distribution and LDH characteristics.

Subjects, Procedures and Methods

16 male physical education students served as subjects. Their mean (\pm S.E.) age, height and weight are 21 yrs, 179 ± 1 cm and 71.3 ± 2.1 kg respectively. Muscle biopsies (Bergström 1962) are taken from the vastus lateralis of the left leg at rest. Histochemical staining for myofibrillar ATPase was undertaken after preincubation at pH 10.3, 4.6 and 4.3 according to Brooks and Kaiser (1970) in order to classify muscle fibres as fast-twitch (FT) and slow-twitch (ST) fibres respectively and in order to separate the FT fibres into FT₁ and FT₂ subgroups. Muscle fibre area is determined according to Thorstensson (1976) from transverse muscle sections stained for NADH dehydrogenase activity according to Nordhoff *et al.* (1961).

The subjects performed 11 repeated maximal isometric leg extensions at a velocity corresponding to 3.14 rad \cdot s⁻¹ so as to induce substantial local muscle fatigue (Thorstensson 1976). The exercise was performed with the subject seated in a fixed position with his leg attached to the lever arm of an isokinetic dynamometer (Cybex II, Laxco Inc., New York) as described elsewhere (Thorstensson 1976). During this type of exercise, EMG recordings have shown that both medial and lateral parts of the vastus muscle are heavily activated (Merrifield and Dostal 1977). In order to ensure that subjects actually started maximal force, 3 single leg extensions are performed two minutes prior to the experiment. The mean force exerted in single extension was performed equivalent to 161.6 Newtonmeter (Nm) compared to 160.4 Nm in the experimental situation.

Immediately after (within 3-4 s) the 25th leg extension, muscle biopsy is taken from the vastus lateralis, frozen in liquid nitrogen and stored at -80°C until analysed. Muscle biopsies were then freeze-dried, and approximately 100 fibres were dissected out (21°C and 50% humidity) and separated into FT and ST fibres on the basis of myofibrillar ATPase staining following preincubation at pH 10.3 according to Essén *et al.* (1975). Pooled ST and FT fibres respectively consisting of 3-10 fibre fragments per pool were weighed on a Cahn electro-balance (Karlsson 1971). The samples ranged 0.01-0.03 g in weight. The lactate concentration is determined by fluorometric means (Karlsson 1971). Each value is then calculated as mean value for the lactate concentration in 2-7 pools of the respective fibre type. All values are converted into wet weight values. A wet content of 77% is assumed for muscle biopsy specimens (Karlsson 1971).

Additional directed pools of FT and ST fibres consisting of about 100 fibres each were homogenized in 100 μ l of 0.5 M KCl. Total LDH activity in the forward reaction (pyruvate \rightarrow lactate, LDH_{total}) is in the backward, diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5, as determined by fluorometric means according to Levy and Provance (1971). Diselectrophoresis for separating the LDH isozymes is performed according to Durr and Labrosse (1967) on 25 μ l of the homogenate. The relative contribution of muscle-specific LDH isozymes ($\%$ M-LDH) is determined using densitometric scanning techniques on the separated and stained LDH isozyme bands on the gels according to Sjodin (1976). The activity corresponding to the muscle-specific LDH (M-LDH) is then calculated ($\%$ M-LDH LDH_{total}).

Relationship between lactate accumulation, LDH activity, LDH isozyme and fibre type distribution in human skeletal muscle

By

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Abstract

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Lactate concentration, total LDH activity and muscle-specific LDH isozymes were determined in pools of the two main types of human skeletal muscle fibres. Analyses were made from biopsy specimens obtained after intense dynamic exercise lasting approximately 30 s. Lactate concentration, total LDH activity and muscle-specific LDH activity displayed higher average values for FT (fast twitch) fibres than for ST (slow twitch) fibres. In addition, positive correlations were found both between the individual percentage of FT fibres and muscle lactate concentration and between lactate concentration and total LDH activity and muscle-specific LDH activity respectively.

High lactate levels have been demonstrated both after exhaustive isometric exercise (Åstrand *et al.* 1972, Karlsson and Ollander 1972) and maximal dynamic exercise (Karlsson 1971, Knuttgen and Saltin 1972) of brief duration and have been suggested as one possible factor contributing to muscle fatigue in maximal exercise of brief duration.

When the lactate concentration in different parts of a muscle sample were analysed after maximal exercise, values in the contracting elements were found to be 3-5 times greater than in elements supposed to be non-contracting (Karlsson 1971). Therefore, it may be assumed that the concentration of metabolites found in whole muscle biopsy specimens does not always reflect the true state of lactate accumulation in exercised muscle.

Essén and Häggmark (1975) reported a greater accumulation of lactate in type II (fast twitch, FT) muscle fibres than in type I (slow twitch, ST) muscle fibres after exhaustive isometric exercise. Recent studies of lactate accumulation in different single muscle fibres have indicated that the fibre type distribution of a muscle is important to lactate accumulation in single muscle fibres (Tesch and Karlsson 1977). In other words, a muscle rich in FT fibres displays a greater potential for accumulating lactate than a muscle rich in ST fibres. This difference may be due to differences in the activity of the glycogenolytic system regulating

zymes, i.e. phosphofructokinase (E.C.2.7.1.11), glycogen phosphorylase (E.C.2.4.1.1) and lactate dehydrogenase ((LDH) E.C.1.1.1.27), in the two main muscle fibre types (Eaton *et al.* 1975, Sjödén 1976, Thorstensson 1976). Thus, both LDH activity and the LDH isozyme distribution pattern in an untrained or moderately trained human population were found to be related to the percentage of FT fibres in the muscle (Karlsson *et al.* 1974). A muscle rich in FT fibres displayed greater LDH activity and a larger contribution of muscle-specific LDH (M-LDH) than a muscle rich in ST fibres in which the heart-specific type of LDH (H-LDH) was predominant. The intracellular distribution of these two main LDH isozymes and their kinetic properties differ (for ref. see Sjödén 1976). In normal conditions, the M-LDH isozymes are mainly associated with membranes, such as the sarcoplasmic reticulum. On the other hand, H-LDH fractions have been found in the inner membrane of the mitochondria.

The aim of the present investigation was to study lactate accumulation in the muscles of individuals differing with respect to certain relevant muscle qualities. Therefore, muscle lactate concentration was determined after intense, standardized exercise of brief duration and related to individual muscle fibre type distribution and LDH characteristics.

Subjects, Procedure and Methods

10 male physical education students served as subjects. Their mean (\pm S.E.) age, height and weight were 4.1 yrs, 179.1 cm and 71.1 \pm 2.1 kg respectively. Muscle biopsies (Bergström 1962) are taken from the vastus lateralis of the left leg at rest. Histochemical staining for myofibrillar ATPase is undertaken after postincubation at pH 10.3, 4.6 and 4.3 according to Brooks and Kaiser (1970) in order to classify muscle fibres as fast twitch (FT) and slow twitch (ST) fibres respectively and in order to separate the FT fibres into FT and FTb subgroups. Muscle fibre area was determined according to Thorstensson (1976) from transverse muscle sections stained for NADH dehydrogenase activity according to Merrifield *et al.* (1961).

The subjects performed 25 repeated maximal unilateral leg extensions at a velocity corresponding to 3.14 rad s^{-1} so as to induce substantial local muscle fatigue (Thorstensson 1976). The exercise was performed with the subject seated in a fixed position, his leg attached to the lever arm of an isokinetic dynamometer (Cybex II, Lomax Inc., New York) as described elsewhere (Thorstensson 1976). During this type of exercise, EMG recordings have shown that both medial and lateral parts of the vastus muscle are heavily activated (Merrifield and Dostal 1977). In order to ensure that subjects actually exerted maximal force, 3 single leg extensions were performed a few minutes prior to the experiment. The maximal force exerted here single extension was performed amounted to 161.6 Newtonmeter (Nm) compared to 160.4 Nm in the experimental situation.

Immediately after (steps 3-4) the 25th leg extension, muscle biopsy as taken from the vastus lateralis, frozen in liquid nitrogen and stored at -80°C until analysed. Muscle biopsies were then freeze-dried, and approximately 100 fibres were dissected out (21°C and 30% humidity) and separated into FT and ST fibres on the basis of myofibrillar ATPase staining following preincubation at pH 10.3 according to Eaton *et al.* (1975). Pooled ST and FT fibres respectively consisting of 5-10 fibre fragments per pool were weighed on.

Calc. electron-balance (Karlsson 1971). The samples ranged 0.01-0.03 mg in weight. The lactate concentration as determined by fluorometric means (Karlsson 1971). Each value was then calculated as mean value for the lactate concentration in 2-7 pools of the respective fibre type. All values were converted into wet weight values. A water content of 77% was assumed for muscle biopsy specimens (Karlsson 1971).

Additional dissected pools of FT and ST fibres consisting of about 100 fibres each were homogenized in 100 μ l of 0.5 M KCl. Total LDH activity in the forward reaction (pyruvate \rightarrow lactate, LDH_{tot}) in the homogenate, diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5, was determined by fluorometric means according to Lowry and Passaniti (1972). Densitometry for separating the LDH isoenzymes was performed according to Dertz and Labranco (1967) on 25 μ l of the homogenate. The relative contribution of muscle-specific LDH isoenzymes (\approx M-LDH) was determined using densitometric scanning technique on the separated and stained LDH isoenzyme bands in the gels according to Sjödén (1976). The activity corresponding to the muscle-specific LDH (M-LDH) was then calculated (\approx M-LDH/LDH_{tot}).

TABLE I Mean values and range for lactate concentration, total LDH activity and M-LDH activity in different muscle fibre types and whole muscle respectively

	FT fibres		ST fibres		Difference	Whole muscle	
	Mean	Range	Mean	Range		Mean	Range
Lactate concentration mmol kg ⁻¹ wet weight	21.7	(4.0-31.3)	15.0	(3.4-30.5)	$p < 0.005$	18.5	(3.5-31.1)
LDH _{tot} activity × mmol min ⁻¹ × kg ⁻¹ wet weight × 10 ⁴	1.12	(0.52-1.82)	0.61	(0.33-1.11)	$p < 0.001$	0.90	(0.31-1.63)
M-LDH activity × mmol min ⁻¹ × kg ⁻¹ wet weight × 10 ⁴	0.82	(0.37-1.76)	0.28	(0.10-0.81)	$p < 0.001$	0.60	(0.18-1.50)

The following formula was used in order to express lactate concentration for the entire muscle investigated. Lactate concentration in FT fibres = FT area + lactate concentration in ST fibres × % ST area. LDH activity or M-LDH activity were calculated in the corresponding manner by substituting lactate concentration for LDH activity in the two main fibre types.

Results

The average percentage of FT fibres in the studied subjects was 46.5% FT fibres (range 29-74%). When the FT fibres were subdivided into FTa and FTb fibre groups, the mean values were found to be 23 and 15% respectively of the total number of fibres. 3 per cent of the fibres were classified as unidentified. The mean relative area taken up by FT fibres amounted to 62.0% (range 28-76%).

Lactate concentration after exercise averaged 21.7 and 15.0 mmol kg⁻¹ wet muscle in FT fibres and ST fibres respectively. There was a considerable inter individual spread in terms of the lactate concentration in both FT (4.0-31.3 mmol kg⁻¹) and ST fibres (3.4-30.5 mmol kg⁻¹) (Table I). The lowest lactate concentration, irrespective of muscle fibre type, was observed in the subject with a muscle richest in ST fibres, whereas the highest lactate concentration observed was found in a muscle consisting of predominantly FT fibres.

The whole muscle values calculated for lactate concentration, LDH_{tot} and M-LDH activity

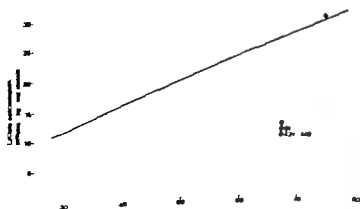


Fig. 1 The relationship between muscle lactate concentration (lactate concentration in FT fibres × FT area + lactate concentration in ST fibres × ST area) and individual muscle fibre type distribution (= FT).

TABLE II. Correlation coefficients and levels of significance for some variables investigated.

	Correlation coefficient	Level of significance
lactate concentration in muscle—muscle fibre type distribution	-0.75	$p < 0.01$
lactate content in FT fibres—muscle fibre type distribution	-0.91	$p < 0.001$
lactate content in ST fibres—muscle fibre type distribution	-0.04	n.s.
lactate concentration in muscle—LDH _{tot} activity	0.66	$p < 0.05$
lactate concentration in muscle—M-LDH activity	0.70	$p < 0.02$
LDH _{tot} activity—muscle fibre type distribution	0.96	$p < 0.001$
M-LDH activity—muscle fibre type distribution	-0.96	$p < 0.001$
M-LDH in FT fibres—muscle fibre type distribution	0.74	$p < 0.01$
M-LDH in ST fibres—muscle fibre type distribution	-0.99	$p < 0.001$

were 18.5 (range 3.5–31.1) mmol kg⁻¹, 0.90 (range 0.38–1.65) and 0.60 (range 0.18–1.54) mmol kg⁻¹ min⁻¹ 10⁻³ wet weight (Table I).

Positive correlations were present between individual muscle lactate concentrations and individual muscle fibre type distribution (Fig. 1, Table II) as well as between individual lactate concentration and individual LDH_{tot} activity and M-LDH activity (Fig. 2, Table II). In agreement with earlier studies (Karlsson *et al.* 1974), positive correlations were found for individual LDH_{tot} activity and M-LDH activity versus individual fibre type distribution (Table II). The relative distribution of M-LDH in both FT and ST fibres was also correlated to the individual muscle fibre type distribution (Fig. 3, Table II).

No significant differences in lactate accumulation were found in subjects with approximately the same percentage of FT fibres but a different FTa/FTb ratio.

Lactate concentration (mmol kg⁻¹ wet weight) in FT fibres % FT area correlated strongly to % FT whereas no corresponding correlation was found between lactate concentration in ST fibres % ST area and % FT (Fig. 4, Table II).

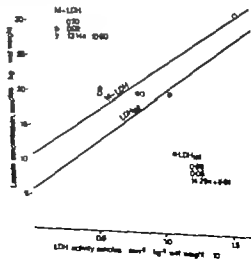


Fig. 2. The relationship between muscle lactate concentration and LDH_{tot} and M-LDH activity respectively.

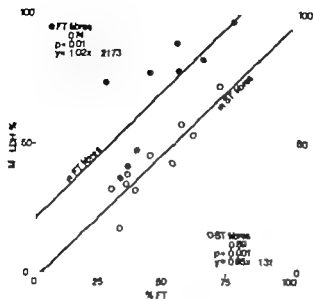


Fig. 3 The relative distribution of M-LDH in FT and ST fibres respectively in relation to individual muscle fibre type distribution (% FT).

Discussion

In the present study a considerable accumulation of lactate was observed in the exercised muscle after only about 30 s. The lactate was not uniformly distributed in the entire exercised muscles, as has been noted elsewhere (Karlsson 1971). The results agree with data obtained from expts. on muscles with different muscle fibre types in rat (Beatty *et al.* 1963). As demonstrated in Fig. 4 there was a positive relationship between the lactate content in the FT fibre portion of the muscle and the percentage of FT fibres in the whole exercised muscle. Corresponding negative relationship did not exist for the lactate content in the ST fibre portion and the percentage of FT fibres, which could be expected assuming that the different fibre types form the same amounts of lactate.

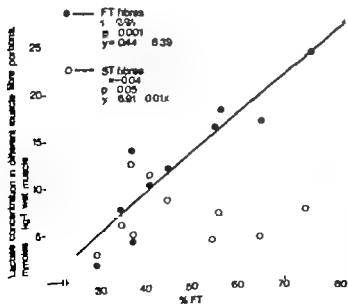


Fig. 4 Lactate content in FT (lactate concentration in FT fibres \times FT area) and ST (lactate concentration \times ST fibres \times ST area) fibre portions of the muscles respectively in relation to individual muscle fibre type distribution (% FT).

The individual differences with respect to lactate accumulated in the muscles after exercise are found to be related to individual variations in percentage of FT fibres, total LDH activity and LDH isozyme pattern. Thus, muscles which had produced high lactate concentrations displayed higher percentage of FT muscle fibres and had a high total LDH and M-LDH activity. The M-LDH isozymes have a higher specific activity and are known to be more efficient in the lactate formation processes as compared to the H-LDH isozymes.

From training studies (Sjodin *et al.* 1976) and from studies on tissue cultures (Dawson *et al.* 1964) it might be established that the conditions in the entire muscle, aerobic or anaerobic, will influence the LDH isozyme pattern in the single muscle cells. Aerobic conditions have been shown to inhibit the M-LDH subunit production while the opposite was true during anaerobic conditions. Due to a lower capillary density in a muscle rich in FT fibres (Andersen, 1975), the conditions have to be more anaerobic than in a muscle rich in ST fibres. Therefore, it was not surprising findings that the relative M-LDH activity in single FT and ST fibres respectively was related to the percentage of FT fibres in the different muscles examined (Fig. 3).

Thus, the consistent pattern with higher lactate concentrations in FT fibres than in ST fibres may be due to the different metabolic profiles of the two main muscle fibre types (Goldstick *et al.* 1974). Support for this view was provided by the present LDH and lactate data.

No lactate analyses were performed on the different subgroups of FT fibres. Consequently it is impossible to determine whether the lactate concentration was more pronounced in one FT subgroup or the other. On the basis of differences in enzymatic profiles, the FTb fibre type might be expected to be better at forming lactate, as noted by Essén *et al.* (1975). However, no support for any such assumption was found when subjects with similar muscle fibre type distribution but different FTa/FTb ratios were compared.

The strong correlation between individual muscle fibre type distribution, expressed as percentage of FT fibres, and both total LDH activity and the relative contribution of M-LDH as found in previous studies (Sjodin 1976) was confirmed. Differences observed in total LDH activity, M-LDH and H-LDH respectively between the two main muscle fibre types also confirmed previous findings (Sjodin 1976).

In summary, data obtained in the present study confirmed previous findings indicating different metabolic properties for the two main skeletal muscle fibres. Thus, fast twitch muscle fibres, characterized by great LDH_{tot} activity and a high proportion of M-LDH, are better at forming lactate than slow twitch muscle fibres.

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Isometric strength performance and muscle fibre type distribution in man

By

PETER TENCH and JAN KARLSSON

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Abstract

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Maximal isometric one-leg strength (MIS) was determined in 34 physical education students displaying wide ranges in muscle fibre type distribution (21.79% fast twitch (FT) muscle fibres) in their vastus lateralis muscles. A linear positive correlation was found between MIS and the relative distribution of FT fibres ($p < 0.001$). This should mean that not only muscle mass involved but also the quality of muscle will be decisive for the ability to develop high isometric strength.

The maximal strength of a muscle has been shown to be proportional to the cross-sectional area of the muscle (Åkai and Fukunaga 1966). In accordance with this, some authors have reported that athletes trained for extreme strength, such as weight-lifters, displayed larger muscle fibres than untrained subjects (Edström and Ekblom 1972, Gollnick *et al.* 1972, Prince *et al.* 1976, Häggmark *et al.* 1978). Häggmark *et al.* also found a correlation between muscle fibre area and the muscle area determined by means of tomography. The "hypertrophy" was predominantly found in the fast twitch (FT or type II) muscle fibres. FT fibre hypertrophy was also noted after 8 weeks of strength training (Thorstensson 1976).

Animal experiments have indicated that white muscles are better adjusted to exerting force than red muscles, even during isometric conditions (Eckes and Sherrington 1930, Burke and Edgerton 1975).

EMG studies with human subjects have disclosed that fast twitch motor units are mainly recruited at high muscle tensions (Gydikov and Kosarov 1974, Burke and Edgerton 1975). Häkkinen *et al.* (1975) found a relationship between the individual relative percentage of slow twitch (ST) fibres and the duration of maximal isometric endurance in two-leg exercise, but they were unable to relate two-leg maximal isometric strength (MIS) to individual muscle fibre type distribution.

It was recently shown in humans that strength output during one-leg extension in a Cybex apparatus (Lumex Inc., New York) at a relatively fast rate of isokinetic muscle

TABLE 1 Anthropometric data, muscle fibre type distribution and muscle strength for the group and subgroup investigated. Values are presented as mean \pm S.E. and range.

	Mean		\pm S.E.		Range	
	n=31	n=25	n=31	n=25	n=31	n=25
Age	25	25	0.8	0.8	20-38	20-35
Height	180	180	0.9	1.1	171-194	171-194
Weight	72	72	1.1	1.3	63-89	63-89
% FT	50	52	2.4	2.7	21-79	21-79
MIS, N	1160	1165	38.0	40.6	736-1595	796-1580
MIS, N kg ⁻¹ b.w.	16	16	0.5	0.5	11-22	12-22

contraction depends on the relative distribution of FT muscle fibres (Thorstensson 1970). He was unable to find any such relationship for either slow isokinetic contractions or isometric contractions. In view of this and the reported suggestion that the muscle fibre recruitment pattern may differ when one- and two-leg exercise was compared (Secher *et al.* 1977), the present investigation was undertaken in order to study one-leg MIS using the same experimental model as Hultén *et al.* (1975).

Material and methods

31 male physical education students volunteered for this study. All subjects were moderately trained, well-trained, but no one was a regular participant in strength- or sprint training. Age, height and weight averaged 25 yrs, 180 cm and 72.3 kg respectively (Table 1).

Biopsies (Bergström 1962) were obtained from the vastus lateralis muscle of the left leg. Muscle samples were mounted in embedding medium, frozen in liquid nitrogen cooled isopentane and stored at -80°C until analysed. Cross-sections were histochemically stained for myofibrillar ATPase after preincubation at pH 10.3 (Padykula and Herman 1955). Muscle fibres were identified as slow twitch (ST) and fast twitch (FT) fibres respectively and the relative distribution of each fibre type was then calculated according to Gollnick *et al.* (1972). At least 700 fibres were counted from each sample.

Transverse serial sections were cut from specimens taken from 25 of the subjects, stained for NADPH diaphorase according to Novikoff *et al.* (1961) and used for calculation of muscle fibre area (Thorstensson 1976). Muscle biopsies were taken a few days prior to strength measurements, made during maximal isometric leg extensions with the left knee at an angle corresponding to 90° . Force was measured as a strain gauge located on an immovable iron bar under the subjects' arches. The experimental setup has been described in detail elsewhere (Karlsson and Ollander 1972, Hultén *et al.* 1975). Conventional statistical methods were employed to calculate mean values, standard error of the mean (S.E.) and linear regression coefficient (r).

Results

Muscle fibre type distribution averaged $50.4\% \pm 2.4$ (range 21-79) FT fibres in the present group (Table 1). Mean maximal isometric strength (MIS) was 1160 Newton (N) or 16.1 N kg⁻¹ body weight (Table 1). Practically the same values were obtained for strength performance and muscle fibre type distribution in the subgroup in which muscle fibre area was determined and relative FT fibre area calculated (Table 1). The relative FT fibre area value obtained in this subgroup amounted to $57.0\% \pm 2.74$ (range 28-77).

A correlation ($p < 0.001$ and $p < 0.01$ respectively) between either muscle fibre type distribution (% FT fibres) or relative muscle fibre area (% FT area) and one-leg MIS

MAXIMAL ISOMETRIC STRENGTH, N
1600 -

1200

800 -

0 20 40 60 80

MUSCLE FIBRE DISTRIBUTION
(% FAST TWITCH FIBRES)

$$y = 9.8x + 725.9$$

$$r = 0.55$$

$$p < 0.001$$

Fig. 1 The relationship between muscle fibre type distribution (FT fibres) and maximal isometric one-leg strength (N).

and Table II) was found. When muscle strength was corrected for body weight, the correlation declined somewhat ($p = 0.01$ and $p = 0.05$ respectively) for both these relationships (Table II).

Discussion

On the basis of histochemical PAS stainings, Gotnick *et al.* (1974) and Secher and Nygaard-Jensen (1976) suggested that FT fibres are mainly recruited during maximal isometric voluntary contractions. The circumstance that FT fibres are activated at high relative isometric tensions, also demonstrated by Gydlkov and Komarov (1974), leads to the hypothesis that a muscle predominantly consisting of FT fibres is better adapted to the development of high tensions than a muscle rich in ST fibres.

The present results appear to confirm the hypothesis. However the results failed to agree with the findings in a previous study based on the same experimental protocol but performed with two-leg exercise (Hultén *et al.* 1975). By way of contrast, Komi *et al.* (1977) were successful in correlating two-leg MLE to the relative distribution of FT fibres in top athletes, including athletes trained for strength and endurance. However these two athletic extremes did differ in respect to muscle fibre type distribution. Thus, athletes representing 'power events' were characterized by a high proportion of FT muscle fibres, whereas the muscles of endurance athletes were made up of predominantly ST fibres. However the

TABLE II Correlation coefficients for variables of significance.

		Correlation coefficient	Level of significance
MLL N vs. FT	31	0.55	$p < 0.001$
MLL N kg^{-1} b.w. vs. FT	31	0.46	$p = 0.01$
MLL N vs. FT arm	25	0.58	$p = 0.01$
MLL N kg^{-1} b.w. vs. FT arm	25	0.49	$p = 0.05$

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Transverse serial sections were cut from specimens taken from 25 of the subjects, stained for NADH diaphorase according to Novikoff *et al.* (1961) and used for calculation of muscle fibre area (Thorstensson 1976). Muscle biopsies were taken a few days prior to strength measurements, made during maximal isometric leg extensions with the left knee at an angle corresponding to 90° . Force was measured in a strain gauge located on an immovable iron bar under the subjects' arches. The experimental setup has been described in detail elsewhere (Karlsson and Olsson 1972, Hultén *et al.* 1975). Conventional statistical methods were employed to calculate mean values, standard error of the mean (S.E.) and linear regression coefficient (r).

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A correlation ($p < 0.001$ and $p < 0.01$ respectively) between either muscle fibre type distribution (% FT fibres) or relative muscle fibre area (% FT area) and one-leg MIS ($F_{1,1}$

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striking difference in the training regimens practiced by these two groups of athletes obscured the relationship between two-leg MIS and individual muscle fibre type distribution. The interpretation of findings is consequently not conclusive.

In addition, information is available pointing to reduced muscle fibre involvement in two-leg exercise compared to one-leg exercise (Secher *et al.* 1977). On the basis of results obtained from experiments in which FT motor units were partially blocked by decamethonium, these authors suggested that fibre recruitment was restricted in two-leg exercise compared to one leg exercise.

When one-leg and two-leg MIS were compared in successful and less successful oarsmen respectively Secher (1975) found that the latter group was incapable of developing more "the one leg force" during two-leg exercise in contrast to the ability of the successful oarsmen. The same pattern was found when body builders and non-strength trained subjects were compared (unpublished observations).

One plausible explanation for this phenomenon is that neuromotor training effects are involved leading to an increased ability to recruit motor units during two-leg exercise. It seems relevant to point out from Secher's two studies that there is some kind of neuromotor blockade during two-leg muscle contraction by untrained subjects. This blockade decreases with strength training. Data obtained by Koml and co-workers (1977) must be interpreted against this background, i.e. their data need not necessarily indicate that subjects rich in FT muscle fibres are stronger.

However, the present data on one leg MIS determinations do lend further support to the view that subjects rich in FT fibres are isometrically stronger. The reason why Thorstensson (1976) was unable to reach the same conclusion is not clear at the moment but may have been related to the different experimental procedure applied.

The greater percentage of FT fibres which was found in the muscles of top athletes participating in "power events" might be the result of selective processes based on the aforementioned relationships between strength performance and muscle fibre type distribution.

To summarize, data were obtained suggesting that muscle fibre type distribution is of importance even to maximal isometric strength performance capacity in addition to its significance in isometric endurance and relatively fast dynamic contractions as previously reported.

This study was supported by grants from the Swedish Medical Research Council (B77-04X-4251-014, B78-04X-4251-05B) and the Research Council of the Swedish Sports Federation.

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The present investigation was undertaken in order to elucidate whether or not beta blockers, when given simultaneously with thyroxine, could antagonize the thyroxine-induced metabolic changes in heart or skeletal muscle (Kubista *et al.* 1971) or in brown adipose tissue (LeBlanc and Villeneuve 1970, Riequier *et al.* 1976, Rabi and Cassuto 1976). The ability of prolonged beta blockade to reduce the enhanced heat production capacity which results from thyroxine injections, was tested by measuring the heat loss of swimming rats (Dawson *et al.* 1970) or the calorogenic response to injected noradrenaline (LeBlanc and Villeneuve 1970).

Baterial and methods

Adult male wistar rats of the Wistar/Kij/Han/Han 67) strain with an average weight of 200 g at the beginning of the experiment were used in these studies. The first group was composed of 18 control animals, injected daily with 0.3 ml of physiological saline-glycerol mixture (1:2). The second group (14 rats) injected daily with 30 µg l-thyroxine/kg, the third group of 4 rats injected daily with 20 mg alprenolol/kg, and finally the fourth group composed of 18 rats, injected daily with 30 µg l-thyroxine + 20 mg alprenolol/kg. The drugs were dissolved in physiological saline and were injected in saline-glycerol (1:2) mixture subcutaneously. The injections were given five times per week for 5 weeks. The animals were kept at room temperature (20-21°C) during the drug regime. 24 h after the 20th drug injection the colonic temperature response to noradrenaline (0.5 mg/kg i.p.) was determined at thermocutaneous temperatures of 29°C (LeBlanc and Villeneuve 1970). Furthermore, the cooling rate of the rats when swimming in water at 25°C was measured (Dawson *et al.* 1970). Colonic temperatures were obtained with a thermocouple inserted to a depth of 4 cm, and recorded on an Elab TE 3 (Copenhagen) potentiometer. This test was performed 24 h after the 22nd drug injection.

The rats were killed 24 h after the last injection by decapitation. Heart, adrenals and interscapular brown adipose tissue (ISBAT) were removed and weighed. Samples of the tip of the myocardium, of the thigh (M. vastus lateralis) muscle and of the ISBAT were immediately frozen in liquid nitrogen, and stored at -80°C until assayed. The tissue samples were homogenized in a Potter-Elvehjem glass homogenizer in Tris-HCl buffer (0.1 M, pH 7.0) to 2% (w/v) homogenate and centrifuged for 10 min at 1000 g at 4°C to remove unbroken cells and peroxide debris. The supernatants were used for the determination of the activities of succinate dehydrogenase (SDH) (E.C. 1.3.99.1) (Earl and Korner 1965), malate dehydrogenase (MDH) (E.C. 1.1.1.37) (Ochoa 1955), citrate synthase (CS) (E.C. 4.1.3.7) (Stern 1962), phosphofructokinase (PFK) (E.C. 2.7.1.11) (Blouin *et al.* 1974), and hexokinase (HK) (E.C. 2.7.1.1) (Bess *et al.* 1969). The activity of lactate dehydrogenase (LDH) (E.C. 1.1.1.37) is determined using commercial reagents (Boehringer Mannheim). The protein concentrations of homogenates are estimated by the phenol method (Lowry *et al.* 1951). For the determination of glycogen content, the muscle samples were homogenized in 1 M-HCl and heated at 100°C for 2 h. The glycogen content was then analyzed as glucose units according to the method of Hultman (1959). Measurements were made at 37°C with Cary 118 double-beam spectrophotometer. The spectra of the four animal groups were always performed together.

Samples of ISBAT were fixed as 10 per cent formal. After dehydration with alcohol and embedding in paraffin, the 7 µm sections were stained with hematoxylin-eosin. The density of fat cell nuclei was calculated from photomicrographs.

Results

The results in Table I show that repeated thyroxine injections retarded the weight gain of the rats. Furthermore the heart, adrenals and ISBAT were larger in thyroxine-treated rats than in the controls. This increased size of ISBAT was not due to hyperplasia of the tissue but most probably to increased fat content of the tissue.

Histological observation of the tissue revealed that the color of the tissue was pale and the fat droplets inside the fat cells were mostly large and unilocular and the density of fat cell nuclei was decreased (Table I). All these changes were observable also in the animals having

Alprenolol fails to antagonize the metabolic changes following repeated thyroxine injections in the rat

By

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Abstract

HARRI, M. N. E. *Alprenolol fails to antagonize the metabolic changes following repeated thyroxine injections in the rat* Acta physiol scand 1978 103 52-58

Repeated injections of rat with 1 thyroxine (50 µg/kg daily for 5 five-day weeks) retarded the weight gain of the animals and increased the absolute and relative size of the heart, adrenals and interscapular brown adipose tissue. In the myocardium and thigh muscle, thyroxine treatment resulted in elevated activity of oxidative enzymes, succinate dehydrogenase, malate dehydrogenase and citrate synthase, while the activities of glycolytic enzymes remained unchanged. Glycogen content of the heart was decreased following thyroxine regime. In the brown fat, on the other hand, thyroxine injections resulted in a reduction of the activity of oxidative enzymes. This reduction can be accounted for by the decreased protein (enzyme) content of the tissue due to deposition of fat. Furthermore, thyroxine treatment delayed the body cooling of the rats swimming in water at 25°C and enhanced hyperthermic response to injected noradrenaline. All these changes, which were not observable in rats treated with daily alprenolol (20 mg/kg) injections, were as pronounced in rats injected with alprenolol together with thyroxine as in rats injected with thyroxine only. It is concluded that beta blockers do not antagonize the metabolic changes due to hyperthyroidism.

Key words: Hyperthyroidism, metabolic changes, body cooling, non-shivering thermogenesis, myocardium, skeletal muscle, brown adipose tissue, beta blockers.

Although the beta adrenergic blocking agents have been employed with satisfactory results in the treatment of the many clinical manifestations of hyperthyroidism (Riddle and Schwartz 1970, Grossman *et al.* 1971 a, b) the mechanism for this action is uncertain. Beta blockers apparently do not influence the thyroid gland or the peripheral metabolism of thyroxine (Azizi *et al.* 1974). There are, however, newer data according to which beta blockers could influence metabolism of thyroxine (Theilade *et al.* 1977). Beta blockers did not abolish the metabolic changes due to experimental hyperthyroidism in the guinea pig (Otter *et al.* 1974). However, beta blockers reduce the response of body temperature, blood free fatty acids or lactate to exercise in hyperthyroid dogs (Kaciuba-Uściłko *et al.* 1976). Furthermore, reduced beta adrenergic responsiveness has been reported in hypothyroid rats (Fregly *et al.* 1977). There is also evidence that prolonged alprenolol treatment could decrease the responsiveness of beta receptors in the rat (Harri 1977).

The present investigation was undertaken in order to elucidate whether or not beta blockers, when given simultaneously with thyroxine, could antagonize the thyroxine induced metabolic changes in heart or skeletal muscle (Kubota *et al.* 1971) or in brown adipose tissue (LeBlanc and Vilmann 1970, Ricquier *et al.* 1976, Rabl and Cassuto 1976). The ability of prolonged beta blockade to reduce the enhanced heat production capacity which results from thyroxine injections, was tested by measuring the heat loss of swimming rats (Dewson *et al.* 1970) or the calorogenic response to injected noradrenaline (LeBlanc and Vilmann 1970).

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TABLE I Effect of daily (for 25-26 d) injections of alprenolol (20 mg/kg), L-thyroxine (30 µg/kg) or their combination on body and organ weight and on relative density of nuclei in histological sections of ISBAT

	Control (18)	Thyroxine (14)	Thyroxine + Alprenolol (15)	Alprenolol (4)
Initial body wt, g	702 ± 5.29	210 ± 7.27	216 ± 6.93	206 ± 2.99
Δ Body wt, g	156 ± 4.14	14 ± 2.85	135 ± 3.34	145 ± 2.48
Heart, mg	825 ± 20.2	850 ± 28.6	792 ± 15.1	728 ± 15.7*
Heart, mg/100 g	270 ± 4.4	300 ± 9.8*	299 ± 4.7*	250 ± 7.0*
Adrenals, mg	55.1 ± 2.15	67.9 ± 4.47*	73.1 ± 2.61	60.8 ± 2.99
Adrenals, mg/100 g	18.0 ± 0.57	24.1 ± 1.87*	27.6 ± 1.28	20.9 ± 0.84*
ISBAT, mg	377 ± 23.7	482 ± 25.8	466 ± 26.7	237 ± 18.7*
ISBAT, mg/100 g	122 ± 4.9	171 ± 10.6	177 ± 12.3	8 ± 3.7***
Nuclei/mm ² in ISBAT	2 162 ± 61	1 610 ± 90*	1 585 ± 88*	2 644 ± 155*

ISBAT = interscapular brown adipose tissue.

Number of rats in parentheses. Significant difference from the controls * $p < 0.05$ ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

received alprenolol injections together with thyroxine, in spite of the fact that alprenolol treatment alone decreased the size of the heart and ISBAT and increased the density of fat cell nuclei in the brown fat. The adrenals were enlarged in all drug treated animals.

The results in Table II show the enzymatic changes due to drug treatment. In the myocardium, thyroxine injections increased the activities of oxidative enzymes, MDH and CS. Other enzymes studied did not show any significant changes. Alprenolol injections, when given together with thyroxine, did not abolish these changes: the increase in CS activity was as great as in animals injected with thyroxine only. The increase in the activity of MDH did not attain the level of statistical significance, due to great individual variation. A decrease in the heart muscle glycogen content was seen both in animals treated with thyroxine only and in animals injected with alprenolol in combination with thyroxine. No significant changes were observable in animals having received alprenolol injections only.

The thigh muscle demonstrated increased activity of oxidative enzymes, SDH, MDH and CS in both thyroxine-treated groups. No other changes were found.

In the ISBAT, on the other hand, a decrement in the activity of the oxidative metabolism was noted. The decrement in the activity of SDH was statistically significant in both thyroxine treated groups and that of MDH and CS in the animals treated with thyroxine only, although a similar tendency was observable in animals having received alprenolol injections in combination with thyroxine. The protein content of the tissue was again decreased in both thyroxine-treated animal groups. A tendency of the increased activity of oxidative enzymes and protein content was evident in rats having received alprenolol injections only. Due to small number of rats, however, these changes did not attain the level of statistical significance (cf. Harri 1977).

Fig. 1 shows the rate of cooling of rats swimming in water at 25°C. The similarities in the initial slope of the lines indicate that the initial rate of cooling was identical in all groups and corresponds to a cooling constant of 4.13 °C/h when calculated according to the formula of $C = 2.3 d \log (T_B - T_A)/dt$ (Morrison and Tice 1957) where T_B and T_A refer to body and

TABLE II Effect of daily (for 25-26 days) injections of alprenolol (20 mg/kg), L-thyroxine (30 µg/kg) and their combination on enzyme activities and protein and glycogen concentrations of rat tissues.

	Control	Thyroxine	Thyroxine + Alprenolol	Alprenolol
Intestine				
Succinate dehydrogenase	39.1 ± 1.01	39.0 ± 0.80	39.1 ± 1.03	37.3 ± 1.76
Malate dehydrogenase	679 ± 12.5	722 ± 9.53	719 ± 16.3	696 ± 33.9
Citrate synthase	113 ± 1.83	122 ± 2.00**	122 ± 1.87**	112 ± 0.93
Phosphofructokinase	47.8 ± 4.18	41.4 ± 9.94	56.2 ± 6.94	44.4 ± 6.30
Hexokinase	3.43 ± 0.16	3.33 ± 0.23	3.06 ± 0.21	3.02 ± 0.24
Lactate dehydrogenase	891 ± 21.9	931 ± 37.3	894 ± 9.55	872 ± 16.1
Protein	88.6 ± 1.05	92.6 ± 0.40	89.7 ± 1.45	89.1 ± 1.25
Glycogen	4.18 ± 0.33	2.57 ± 0.35**	2.96 ± 0.16**	3.22 ± 0.11
Liver				
Succinate dehydrogenase	6.22 ± 0.22	6.99 ± 0.25*	6.98 ± 0.38*	5.77 ± 0.34
Malate dehydrogenase	188 ± 4.77	200 ± 5.33	214 ± 12.7*	180 ± 1.6
Citrate synthase	26.2 ± 0.30	27.3 ± 0.94	31.2 ± 1.86	26.0 ± 1.19
Phosphofructokinase	16.1 ± 1.23	18.7 ± 2.44	17.6 ± 1.40	18.5 ± 2.56
Hexokinase	3.12 ± 0.21	3.72 ± 0.23	3.81 ± 0.39	3.10 ± 0.57
Lactate dehydrogenase	1 000 ± 24.8	1 084 ± 31.7	1 060 ± 20.7	1 036 ± 47.4
Glycogen	5.20 ± 0.32	4.54 ± 0.32	5.40 ± 0.37	5.19 ± 1.10
ISBAT				
Succinate dehydrogenase	23.4 ± 1.17	19.0 ± 0.84**	17.9 ± 0.72**	23.2 ± 1.76
Malate dehydrogenase	449 ± 24.1	377 ± 16.5**	409 ± 31.7	473 ± 30.3
Citrate synthase	69.3 ± 7.60	48.7 ± 4.79*	54.4 ± 4.82	83.3 ± 8.47
Hexokinase	1.39 ± 0.23	1.16 ± 0.14	1.13 ± 0.17	~
Lactate dehydrogenase	234 ± 37.2	209 ± 19.6	229 ± 41.5	266 ± 18.7
Protein	73.8 ± 3.00	59.5 ± 3.01	57.8 ± 2.70**	79.5 ± 1.85

ISBAT: interscapular brown adipose tissue. The number of rats identical to those given in Table I. Enzyme activities are expressed as μ moles of substrate utilized per min per g wet weight, and protein and glycogen as mg/g wet tissue weight. Significant differences from the controls: *p 0.05, **p 0.01 and ***p 0.001 (Student's t-test).

ambient temperature, respectively. However later the rate of cooling slowed down in the thyroxine-treated groups, the time of the T_{re} to reach 30°C was significantly longer both in the animals treated with thyroxine only and in animals having received alprenolol injections in combination with thyroxine than in the control rats or in the rats treated with alprenolol only.

Fig. 2 shows the metabolic response of the rats to injected noradrenaline (0.5 mg/kg i.p.). In both thyroxine-treated groups noradrenaline induced a significant rise in the colonic temperature, while in other groups it failed to cause hyperthermia.

Discussion

In accordance with earlier findings, the heart and ISBAT were smaller in alprenolol-treated rats than in the controls (Harr 1977). In spite of that, the organ weights in rats treated with alprenolol in addition to thyroxine more closely resembled those of the rats injected with thyroxine only. Enlargement of the adrenals in all drug-treated animals can be regarded as an indication of non-specific stress resulting from the change of drugs. It is interesting to note that this enlargement was greatest in animals having received both alprenolol and thyroxine injections than in animals subjected to one drug only.

The unchanged activity of some enzymes in rats treated with alprenolol only (at a room

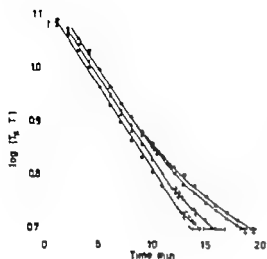


Fig. 1 The rate of body cooling in rats withdrawing a water at 25°C. ● = control rats, Δ = alprenolol-treated rats (20 mg/kg daily for 5 five-day weeks), ○ = thyroxine-treated rats (50 μg/kg daily for 5 five-day weeks), and × = rats injected with alprenolol together with thyroxine. T_b , T_a = the difference of body and ambient (water) temperature. The time of the body temperature to cool to 30°C is indicated at the end of each line as the mean ± S.E. The broken lines extrapolate the initial rates of body cooling — significantly different from the controls ($p < 0.001$ Student's *t*-test). The rats per group.

temperature of 20–21°C) is not only due to a small number of rats in this group but is in accordance with an earlier more detailed investigation (Harri 1977).

The enzymatic responses of the heart and skeletal muscles to thyroxine were relative small and they were seen in the oxidative enzymes only. This is not very surprising because in an earlier study the enzymatic changes even to massive dosage of thyroid hormone did not reach more pronounced values (Kubista *et al.* 1971). The increment in the activity of Hk, which was very marked in the study of Kubista *et al.* 1971, was not statistically significant in the present study. The response of Hk to a single exercise bout (Barnard and Peter 1969, Boström *et al.* 1974) indicates the acute nature of the response. It is thus possible that the response of Hk to a few injections of massive doses of thyroxine (Kubista *et al.* 1971) is not comparable to the response observed in this study as a result of prolonged treatment with a much smaller dosage.

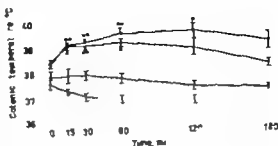
However the most interesting observation of the present study was that alprenolol when given together with thyroxine failed to antagonize thyroxine-induced enzymatic changes.

The significance of the decreased glycogen content in the heart but not in the skeletal muscle in thyroxine-treated rats remains to be clarified by further experiments. The decrease in the glycogen content possibly reflects elevated aerobic carbohydrate catabolism of the myocardium as suggested by Kubista *et al.* 1971. This indicates that the response of the heart muscle to thyroid hormone can be different from that of especially white skeletal muscle (Kubista *et al.* 1971). From the point of view of the present study however it is interesting to note that the glycogen drop was as great in animals having received alprenolol in combination with thyroxine as in animals having received thyroxine injections only.

The metabolic response of ISBAT to thyroxine regime, i.e. the decreased activity of mainly oxidative enzymes was coupled with a decrease in the concentration of protein in the tissue.

Histological observation revealed that the ISBAT from thyroxine-treated rats was pale in color and the fat droplets were large and mostly unilocular. Accumulation of fat in the ISBAT following hyperthyroidism has been reported earlier in hamster (Rabl and Casuso 1976) and rat (Riequier *et al.* 1976). It can thus explain the decreased density of fat cell nuclei and the decreased protein concentration observed in the present study in these animals.

Fig. 2. The response of the body temperature to injected noradrenaline (5 mg/kg p.v.) at 29°C in control rats (●), in alprenolol-treated rats (20 mg/kg daily for 5 five-day weeks) (Δ), thyroxine-treated rats (30 μg/kg daily for 5 five-day weeks) (○), and in rats injected with alprenolol together with thyroxine (□). Vertical bars indicate \pm S.E. Noradrenaline was injected at 0 min. Asterisks mean those hyperthermic changes which significantly differ from the initial value (● only): *p < 0.05, **p < 0.01 and ***p < 0.001. Four rats per group.



The ISBATs of the rats treated with alprenolol in addition to thyroxine more closely resembled those of the animals treated with thyroxine only than those of animals injected with alprenolol only.

When the difference between body and water temperatures is large in a swimming rat, the heat loss by convection greatly exceeds heat production (Dawson *et al.* 1970). In this situation the insulative capacity of the body primarily determines the rate of body cooling. In this study the initial slope of body cooling was very similar in all experimental groups used, an indication that the insulative capacity was about similar in them. Later when the difference between ambient and body temperature becomes smaller the metabolic heat production slows down the rate of body cooling. Thus, the more the rate of body cooling shifts from the initial slope the more metabolic heat production exceeds heat loss.

In this study the rate of body cooling was, in spite of initial identical rate in all groups, significantly slower in the rats treated with thyroxine alone or in combination with alprenolol than in the other groups. Moreover there was neither significant difference between the two thyroxine groups nor between the controls and the rats injected with alprenolol only. Thus a conclusion has to be drawn that alprenolol did not impair the calorigenic effect of thyroxine treatment.

Increased non-shivering thermogenesis, *i.e.* calorigenic response to injected noradrenaline, has been reported to result from prolonged thyroxine treatment (LeBlanc and Videmaire 1970). In this study the increase in colonic temperature after noradrenaline injection was used as a measure of non-shivering thermogenesis. It was observable in rats treated with thyroxine alone as well as in rats having received alprenolol injections in combination with thyroxine but not in other experimental groups. Here again, it can be concluded that alprenolol treatment did not abolish or diminish the enhanced non-shivering thermogenesis due to thyroxine regime.

In conclusion, the present results demonstrate that none of the measured parameters, which are influenced by prolonged thyroxine injections, could be antagonized by alprenolol injections given together with thyroxine. These findings are even more obvious because alprenolol treatment alone either did not induce any changes or caused changes, which were opposite to those of thyroxine. Thus the beneficial effect of beta blockers in the treatment of many clinical manifestations of hyperthyroidism (Ruddle and Schwarz 1970, Grossman *et al.* 1971 a, b) cannot be accounted for by metabolic way of effect.

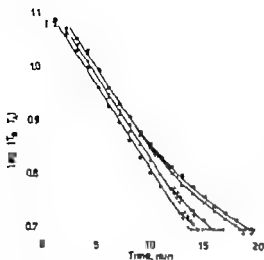


Fig. 1 The rate of body cooling in rats swimming in water at 25°C. ● = control rats, Δ = alprenolol-treated rats (20 mg/kg daily for 5 five-day weeks), ○ = thyroxine-treated rats (50 μg/kg daily for 5 five-day weeks) and × = rats injected with alprenolol together with thyroxine. $T_B - T_A$ = the difference of body and ambient (water) temperature. The time of the body temperature in cool to 30°C is indicated at the end of each line as the mean ± S.E. The broken lines extrapolate the initial rates of body cooling — significantly different from the controls ($p < 0.001$ Student's t -test). Five rats per group.

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However, the most interesting observation of the present study was that alprenolol when given together with thyroxine failed to antagonize thyroxine-induced enzymatic changes.

The significance of the decreased glycogen content in the heart but not in the skeletal muscle in thyroxine-treated rats remains to be clarified by further experiments. The decrease in the glycogen content possibly reflects elevated aerobic carbohydrate catabolism of the myocardium as suggested by Kubista *et al.* 1971. This indicates that the response of the heart muscle to thyroid hormone can be different from that of especially white skeletal muscle (Kubista *et al.* 1971). From the point of view of the present study, however, it is interesting to note that the glycogen drop was as great in animals having received alprenolol in combination with thyroxine as in animals having received thyroxine injections only.

The metabolic response of ISBAT to thyroxine regime, *i.e.* the decreased activity of mainly oxidative enzymes, was coupled with a decrease in the concentration of protein in the tissue.

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On the chemical nature of the blood borne cardiotoxic material released from the feline small bowel in regional shock

By

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Abstract

LUNDGREN, O. and U. HAGLUND. *On the chemical nature of the blood borne cardiotoxic material released from the feline small bowel in regional shock.* Acta physiol. scand. 1978 103: 59-70.

The cardiotoxic material released into blood from the feline small intestine during 2 or 3 h regional shock period (infuse perfusion to the small intestine 30-35 mmHg during continuous activation of the regional sympathetic vasoconstrictor fibres at 6 Hz) has been analyzed with regard to three properties: 1. molecular mass determined by molecular filtration; 2. solubility in monopolar solvent (ether); 3. heat stability by heating plasma to 80°C for 30 min. The results obtained suggest that the cardiotoxic material consists of at least two heat stable fractions. One is water soluble with molecular mass between 500 and 1 000 d and the other lipid soluble with an unknown molecular mass.

It is well established that ischemia in the splanchnic region may produce a shocklike state in an experimental animal (for a recent review see Marston 1977). The pathogenesis of this shock situation is, according to some authors, secondary to the release of toxic material from the hypoxic tissue (for reviews, see Graber 1967 and Lefer 1973). In this laboratory we have demonstrated that simulating a shock situation in the feline small bowel by lowering arterial inflow pressure to 30-35 mmHg for 2 or 3 h during a concomitant activation of the regional sympathetic vasoconstrictor fibres at 6 Hz almost invariably produces tissue damage, particularly at the villous tips. The development of these lesions is, at least in part, due to an exaggerated extravascular shunting of oxygen in the intestinal countercurrent exchanger as result of reduced linear flow rate in the villous vascular loops (cf Haglund *et al* 1973, Haglund and Lundgren 1974). After such a simulated intestinal shock period in an otherwise normotensive animal the cardiovascular system deteriorates, as judged from a rapid blood pressure fall of 40-50 mmHg during the first hour after the regional hypotension (Haglund and Lundgren 1973, Haglund *et al* 1976 b). Perfusing the gut lumen with an oxygenated saline solution prevented the development of the tissue lesions as well as the cardiovascular collapse (Haglund *et al* 1976 a, b). From these and other studies (Haglund and Lundgren 1973) it was inferred that blood borne

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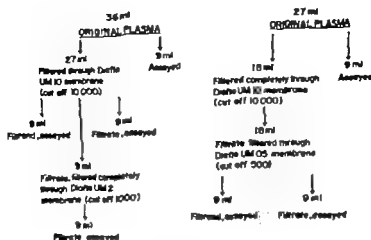


Fig 1 The two filtration procedures used, schematically illustrated.

T. obum control plasma. Identical experiments to those described above were performed except that no regional intestinal shock was induced.

B. Histological technique

30–60 min after the exchange transfusion the small intestine was excised and weighed. 1 cm² x 1 cm² rectangular pieces (about 4 cm) of the small gut wall was cut out and after mounting it on cork, it was fixed in 10% neutral formalin. The tissue specimen was then prepared with routine histological techniques and stained with hematoxylin and eosin. As described earlier (Chen *et al.* 1970, Åhrén and Haglund 1973) the morphological changes in the mucosa were graded into 6 grades, where grade 0 means normal mucosa and 1 to 5 denote increasing damage to the intestinal epithelium. The pathognomonic lesion for grade 1 is the development of subepithelial space at the tip of the villi. This space is more extended in grade 2 where also epithelial lifting is seen. In grade 3 there is massive epithelial lifting also along the sides of the villi and in grade 4 the villi are denuded. Grade 5 is characterized by disintegration of the lamina propria, hemorrhage and sloughing in the mucosa. In the deeper layers of the intestinal wall the tissue is intact.

It is demonstrated in control experiments (Haglund and Lundgren, unpublished observations) that the tissue damage, as judged by the histological appearance, is the same in all parts of a small bowel subjected to periods of regional shock.

C. Biochemical procedures

The different plasma samples are subjected to 3 different procedures, ultrafiltration, ether extraction and heating.

1. *Molecular ultrafiltration.* Control and shock plasma was subjected to two different molecular filtration procedures (Fig. 1), using Daclo molecular filters (manufactured by Amicon Corp., Lexington, Mass.). The filtrations were carried out in magnetically stirred ultrafiltration cell (Amicon Model 52) at 20–25°C at pressure of 144 kPa (50 psi). Before each plasma filtration about 50 ml of sterile, pyrogen free, isotonic saline solution was filtered through the membrane to be used. Filtration rate was determined and if the magnitude of this rate deviated markedly from earlier filtrations with the same filter or filters of the same type, the filter was discarded.

The details of the two filtration procedures are depicted in Fig. 1. With the procedure shown in the left part of the figure four plasma samples were obtained: original plasma, UM 10 filtrate (containing solutes with molecular mass above 10 000 d concentrated three times), UM 10 filtrate (solutes with molecular mass below 10 000 d) and UM 2 filtrate (solutes with molecular mass below 1 000 d). Similarly in the other filtration procedure illustrated in the right part of Fig. 1, three plasma solutions were obtained: original plasma, UM 0.5 filtrate (containing solutes with molecular mass between 300 and 10 000 d concentrated two times) and UM 0.5 filtrate (containing solutes with molecular mass below 300 d).

Molecular sieving and ether extraction. In the series of experiments, plasma from control and shocked

material is released from the villous tissue lesions, producing hypotension in the experimental animal.

A negative inotropic influence on the heart constitutes one important mechanism for the development of the shock like state described above. This conclusion is based on *in vivo* studies (Haglund and Lundgren 1973, Haglund *et al.* 1978) as well as on *in vitro* studies using two different myocardial preparations (Lundgren *et al.* 1976). In the latter study it was demonstrated that exposing rabbit papillary muscle to cat plasma, collected in the intestinal vein immediately after a simulated shock period, reduced peak isometric tension to half of control value while control plasma had no such effect. Hence, it was concluded that the feline small intestine in shock produced material with a negative inotropic effect on the heart.

The present study represents an attempt to chemically characterize this material as a first step in an eventual chemical isolation and identification. Three questions have been considered in this study: What is the molecular mass of the material? Is it soluble in nonpolar solvents? Is it heat stable or heat labile?

Methods

A. Experiments on rats

The experimental procedures for the studies on cats have been described in detail elsewhere (Haglund and Lundgren 1973) and only a brief summary is given here. The animals had been deprived of food for 12 h and had no obvious signs of intestinal diseases. They were anesthetized with chloralose (50 mg/kg b.wt. i.v.). A major portion of the small bowel (weight 50–70 g) was isolated and the remainder of the intestine, the spleen, the greater omentum and the major part of the pancreas were eviscerated. The superior mesenteric artery supplying the isolated intestinal segment, was surrounded by an adjustable clamp so that the regional arterial inflow pressure could be lowered to any desired level. The nerves surrounding the superior mesenteric artery were cut and their distal ends were placed on a ring electrode for electrical stimulation. The left adrenal gland was denervated and the vessels supplying the right adrenal were ligated. Atropine (1 mg/kg b.wt.) was given i.v.

After heparinization, the left femoral artery and a minor branch of the superior mesenteric artery distal to the adjustable clamp were cannulated to record arterial pressure by pressure transducers (Statham P23). The mesenteric vein was cannulated and the venous outflow from the intestinal segment and its lymph nodes was recorded continuously by an optical drop recorder coupled to a ordinate writer. Venous outflow pressure was set at 10 mmHg. The blood was returned to the animal through a catheter in the jugular vein. Recordings were made on a Grass polygraph.

A slow i.v. infusion of a glucose solution containing bicarbonate (10 mmol of N HCO_3 per 100 ml of 10% glucose solution, given at a rate of 0.1–0.2 ml/min) was started on induction of anesthesia and continued throughout the expt. This infusion has been shown to maintain arterial pH at normal level, despite the operative trauma and intestinal hypotension (Haglund and Lundgren 1972). The tubes and the funnel draining the intestinal venous blood were primed with 6% dextran solution (1/3 low molecular weight dextran (mean molecule mass 40 000 d) and 2/3 medium molecular weight dextran (mean molecular mass 70 000 d) Pharmacia, Uppsala, Sweden). Further 2–3 ml of this solution was given in the animal every 20 min during the hypotensive period.

After completion of the operative procedures a period of 30–40 min was allowed to pass. Then an intestinal shock was mimicked by lowering arterial inflow pressure to 30–35 mmHg during a continuous electrical stimulation of the regional vasoconstrictor fibres at 6 Hz with square wave pulses of 6 ms and 12 V. These manipulations produced a low flow state in the small intestine similar to that seen in severe hemorrhagic shock (Haglund 1973). It should be pointed out that the remaining part of the pancreas is perfused at normal arterial pressure via the celiac artery. Immediately after a 3-hour period of regional shock, the intestinal venous outflow was collected in a cooled plastic beaker for about 5 min (80 ml) with a corresponding substitution of fresh blood from another cat. After centrifugation the plasma ("shock plasma") was stored in plastic tubes at -20°C until tested on the bioassay systems (see Bioassay technique).

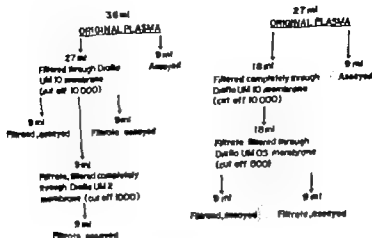


Fig. 1 The two filtration procedures used, schematically illustrated.

The above "control plasma" identical experiments to those described above were performed except that no regional intestinal shock was induced.

B. Histological technique

30–60 min after the exchange transfusion the small intestine was excised and weighed. In most expts. rectangular pieces (about 2–4 cm) of the mid gut wall was cut out and after smoothing it on cork, it was fixed in 10% neutral formalin. The tissue specimens were then prepared with routine histological techniques and stained with hematoxylin and eosin. As described earlier (Cline *et al.* 1970, Åhrén and Haglund 1973) the morphological changes in the mucosa were graded into 6 grades, here grade 0 means normal mucosa and 1 to 5 denote increasing damage to the intestinal epithelium. The pathognomonic lesion for grade 1 is the development of subepithelial spaces at the tip of the villi. This space is more extended in grade 2, here also epithelial lifting occurs. In grade 3 there is massive epithelial lifting also along the sides of the villi and in grade 4 the villi are denuded. Grade 5 is characterized by disintegration of the lamina propria, hemorrhage and ulceration in the mucosa. In the deeper layers of the mucosal wall are intact.

It was demonstrated in control expts. (Haglund and Lindgren, unpublished observations) that the tissue damage, as judged by the histological appearance, is the same in all parts of small bow of subjected to period of regional shock.

C. Biochemical procedures

The different plasma samples were subjected to 3 different procedures, ultrafiltration, ether extraction and heating.

1. *Molecular ultrafiltration.* Control and shock plasmas were subjected to two different molecular filtration procedures (Fig. 1), using Dufflo® molecular filters (manufactured by Amicon Corp., Lexington, Mass.) The filtrations were carried out in magnetically stirred ultrafiltration cell (Amicon Model 52) at 20–22°C in presence of 344 kPa (30 psi). Before each plasma filtration about 50 ml of sterile, pyrogen free, isotonic saline solution was filtered through the membrane to be used. Filtration rate as determined and if the magnitude of this rate decreased markedly from earlier filtrations with the same filter or filters of the same type, the filter was discarded.

The details of the two filtration procedures are depicted in Fig. 1. With the procedure shown in the left part of the figure four plasma samples were obtained: original plasma, UM10 filtrate (containing solutes with molecular mass above 10 000 d concentrated three times), UM10 filtrate (solutes with molecular mass below 10 000 d) and UM2 filtrate (solutes with molecular mass below 1 000 d). Similarly in the other filtration procedure illustrated in the right part of Fig. 1 three plasma solutions are obtained: original plasma, UM05 filtrate (containing solutes with molecular mass between 500 and 100 000 d concentrated two times) and UM05 filtrate (containing solutes with molecular mass below 500 d).

2. *Molecular dialysis and ether extraction.* In this series of expts. plasmas from control and shocked

material is released from the villous tissue lesions, producing hypotension in the experimental animal.

A negative inotropic influence on the heart constitutes one important mechanism for the development of the shock-like state described above. This conclusion is based on *in vivo* studies (Haglund and Lundgren 1973, Haglund *et al.* 1978) as well as on *in vitro* studies using two different myocardial preparations (Lundgren *et al.* 1976). In the latter study it was demonstrated that exposing rabbit papillary muscle to cat plasma, collected in the intestinal vein immediately after a simulated shock period, reduced peak isometric tension to half of control value while control plasma had no such effect. Hence, it was concluded that the feline small intestine in shock produced material with a negative inotropic effect on the heart.

The present study represents an attempt to chemically characterize this material as a first step in an eventual chemical isolation and identification. Three questions have been considered in this study: What is the molecular mass of the material? Is it soluble in nonpolar solvents? Is it heat stable or heat labile?

Methods

A. Experiments on cats

The experimental procedures for the studies on cats have been described in detail elsewhere (Haglund and Lundgren 1973) and only a brief summary is given here. The animals had been deprived of food for 12 h and had no obvious signs of intestinal diseases. They were anesthetized with chloralose (50 mg/kg b.w.). A major portion of the small bowel (weight 50–70 g) was isolated and the remainder of the intestine, the spleen, the greater omentum, and the major part of the pancreas were extirpated. The superior mesenteric artery supplying the isolated intestinal segment, was surrounded by an adjustable clamp so that the regional arterial inflow pressure could be lowered to any desired level. The nerves surrounding the superior mesenteric artery were cut and their distal ends were placed on a ring electrode for electrical stimulation. The left adrenal gland was denervated and the vessels supplying the right adrenal were ligated. Atropine (1 mg/kg b.w.) was given i.v.

After heparinization, the left femoral artery and a minor branch of the superior mesenteric artery distal to the adjustable clamp were cannulated to record arterial pressure by pressure transducers (Statham P23). The mesenteric vein was cannulated and the venous outflow from the intestinal segment and its lymph nodes was recorded continuously by an optical drop recorder coupled to a ordinate writer. Venous outflow pressure was set at 10 mmHg. The blood was returned to the animal through a catheter in the jugular vein. Recordings were made on Grass polygraph.

A slow i.v. infusion of a glucose solution containing bicarbonat (10 mmol of NaHCO_3 per 100 ml of 10% glucose solution, given at a rate of 0.1–0.2 ml/min) was started on induction of anesthesia and continued throughout the experiment. This infusion has been shown to maintain arterial pH at normal level, despite the operative trauma and intestinal hypotension (Haglund and Lundgren 1977). The tubes and the cannula draining the intestinal venous blood were primed with a 6% dextran solution (1/3 low molecular weight dextran (mean molecular mass 40 000 d) and 2/3 medium molecular weight dextran (mean molecular mass 70 000 d) Pharmacia, Uppsala, Sweden). Further 2–3 ml of this solution was given to the animal every 20 min during the hypotensive period.

After completion of the operative procedures, a period of 30–40 min was allowed to pass. Then an 'intestinal shock' was mimicked by lowering regional inflow pressure to 30–35 mmHg during a continuous electrical stimulation of the regional vasoconstrictor fibres at 6 Hz with square wave pulses of 6 ms and 12 V. These manipulations produced a low flow state in the small intestine similar to that seen in severe hemorrhagic shock (Haglund 1973). It should be pointed out that the remaining part of the pancreas is perfused at normal arterial pressure via the celiac artery. Immediately after a 3-hour period of regional shock, the intestinal venous outflow was collected in a cooled plastic beaker for about 5 ml (80 ml) with a corresponding substitution of fresh blood from another cat. After centrifugation the plasma ('shock plasma') was stored in plastic tubes at -20°C until tested on the bioassay systems (see Bioassay techniques).

Summary
 The quantitative effect of any given test solution on the papillary muscle is defined as the ratio (R) of peak isometric tension developed in the test solution (plasma) to that developed in the control bathing modified Krebs-Henseleit solution, i

$$\frac{\text{peak isometric tension, test solution}}{\text{peak isometric tension, control solution}} \quad (1)$$

After this one muscle was used to test each plasma sample, and to calculate the average value the contracture rather than end-diastolic tension is used. Thus, if the isometric tension of two muscles doubled that of two muscles is halved, the geometric mean is 1.00 in contrast to the arithmetic mean of 2.5. The geometric mean isometric tension ratio (\bar{R}) of n muscles is calculated conventionally as

$$\bar{R} = \text{antilog } \frac{1}{n} \sum \log R$$

For statistical purposes $\log \bar{R}$ was used. Statistical significance was calculated by Wilcoxon test for two samples (Dixon and Massey 1970) or by the sign test (Siegel 1956). A p -value of 0.05 or less was judged significant. The significance level of 0.05 was chosen since the sign test gives this p -value when 6 of 7 observations show the same trend (Siegel 1956).

Results

A Experiments on cats

The results of the cat experiments are summarized in Fig. 1. It can be seen that lowering arterial inflow pressure to 30–35 mmHg during a continuous stimulation of the regional sympathetic vasoconstrictor fibres induced an initial pronounced vasoconstriction, intestinal blood flow being reduced to 10–20% of control. Within 20–40 min blood flow had increased to a steady state level of about 25% of control, evidently due to a decrease of regional flow resistance. In the control expts. blood pressure and intestinal blood flow remained fairly constant throughout the expts.

The results of the grading performed on the histological sections of most of the experimental animals are given in Table I. The small bowel of control animals was graded as 0 or 1 while the gut from most shock animals exhibited mucosal lesions rated as grade 4 or 5.

B Experiments on isolated rabbit papillary muscles

I Molecular filtration. The results of the two filtration procedures used are shown in Table II (log \bar{R} values) and III (plasma electrolyte concentrations and time to peak tension). The 4 plasma fractions obtained by filtering through UM10 and UM2 filters (left panel of Fig. 1) were assayed on 30 rabbit papillary muscles (control plasma 122, shock plasma 108). Three plasma fractions were produced by the UM10 and UM05 filtrations (right panel of Fig. 1) and tested on 114 rabbit papillary muscles (control plasma 47, shock plasma 67). All shock plasma fractions except the UM10 filtrate (< 10 000 d) and the UM05 filtrate (> 500 d) differed significantly from the controls (Table II). No statistically significant difference between control and shock plasma samples was found with regard to their electrolyte concentrations (Table IV). Time to peak tension was only slightly affected by the different plasma samples (Table IV).

When comparing the effects on the papillary muscles of the different control plasma fractions with each other it was found that the UM10 filtrate (< 10 000 d) differed significantly

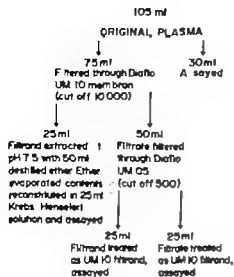


Fig. 1. The filtration procedure employed when performing ether extractions. The original plasma sample consisted of pooled plasma from three cat experiments.

animals were subjected to a filtration procedure depicted in Fig. 2. As illustrated the fractionation procedure produced 4 different plasma fractions: original plasma, UM10 filtrate (concentrated 3 times), UM05 filtrate (concentrated 2 times) and UM05 filtrate. The pH of the 3 last mentioned solutions was adjusted to 7.3 and extracted with a double volume of distilled ether. The ether was evaporated under nitrogen at 30°C and the ether-soluble solutes were dissolved in the original volume of a Krebs-Henseleit buffer solution (see below) by ultrasonic agitation.

3. Heating To determine if the cardiotoxic material was heat stable, original plasma was heated in a water bath to 80°C for 30 min. The plasma was then centrifuged (12 000 *g*) for 10 min at 4°C and the supernatant was tested.

The different solutions obtained at the various biochemical manipulations described above were analyzed with regard to their Na, K and Ca concentrations in an Eppendorf flame photometer. The pH of the test samples was adjusted to 7.35–7.40 when bubbled with 4% CO₂ in O₂. In a few plasma samples the concentration of one of the mentioned ions was increased by addition of proper amounts of a concentrated solution of the chloride salt of the electrolyte. The sensitivity of the present bioassay system for changes in [Na], [K] and [Ca] was tested and reported in a previous study (Lundgren *et al.* 1976).

D. Bioassay technique

Albino Swedish Land rabbits weighing 1.5–2.5 kg were killed by a blow on the neck. The heart was removed rapidly and the right ventricle was opened while immersed in cold oxygenated incubation solution. The papillary muscles were dissected free together with a small piece of the ventricular wall adjacent to the muscle base. The preparation was mounted vertically in a mantled temperature-controlled bath (volume 2.0–2.5 ml) between a fixed hook and a force-displacement transducer (Grass FT 03C). The temperature of the bath was kept at 37°C by thermostat and the solution was continuously gassed with 4% CO₂ in O₂.

After an equilibration period of 30 min the muscle was stimulated at 1 Hz by application of an electrical field between two platinum electrodes. The pulse duration was set at 1 or 2 ms and the stimulus intensity at 50% above threshold. The changes in tension induced by the isometric contractions were recorded on a Grass polygraph. The preparation was first stimulated for at least 1 h. A length-force curve was then determined for each muscle and the length giving 80–90% of the maximal contractile force was chosen as "resting" length for the rest of the experiment. The muscle was allowed to contract for another 30 min before it was exposed to the solution to be assayed. Only one plasma sample was tested on each muscle. After the expts. the length of the muscle was determined *in situ* with a pair of callipers. The wet weight of the muscle was measured on a Cahn, model 11 electrobalance.

E. Solution

The bathing solution used in the papillary expts. was a modified Krebs-Henseleit solution containing (mmol/l): NaCl 120, KCl 3.3, NaHCO₃ 20, KH₂PO₄ 1.2, MgCl₂ 6H₂O 1.2, CaCl₂ 2.5, glucose 11.5.

Contract

quantitative effect of any given test solution on the papillary muscle was defined as the ratio (R) of the isometric tension developed in the test solution (plasma) to that developed in the control bathing liquid Krebs-Henseleit solution, i

$$\frac{\text{peak isometric tension, test solution}}{\text{peak isometric tension, control solution}} \quad (1)$$

One test muscle was used to test each plasma sample, and to calculate the average value the isometric tension of two muscles doubled that of two muscles as below, the geometric mean was 1.00 in contrast to the arithmetic mean of 1.00. The geometric mean isometric tension ratio (\bar{R}) of n muscles was calculated conventionally as

$$\text{antilog } \frac{1}{n} \sum \log R_i$$

For statistical purposes $\log \bar{R}$ was used. Statistical significance was calculated by Wilcoxon's test for n samples (Dixon and Lehner 1975) or by the sign test (Siegel 1956). A p -value of 0.05 or less was judged significant. The significance level of 0.05 was chosen since the sign test gives this p -value when 2 of 7 observations show the same trend (Siegel 1956).

Results

A. Experiments on cats

The results of the cat experiments are summarized in Fig. 3. It can be seen that lowering arterial inflow pressure to 30–35 mmHg during a continuous stimulation of the regional sympathetic vasoconstrictor fibres induced an initial pronounced vasoconstriction, intestinal blood flow being reduced to 10–20% of control. Within 20–40 min blood flow had increased to a steady state level of about 25% of control, evidently due to a decrease of regional flow resistance. In the control expts. blood pressure and intestinal blood flow remained fairly constant throughout the expts.

The results of the grading performed on the histological sections of most of the experimental animals are given in Table I. The small bowel of control animals was graded as 0 or 1 while the gut from most shock animals exhibited mucosal lesions rated as grade 4 or 5.

B. Experiments on isolated rabbit papillary muscles

1. *Stalemler filtration.* The results of the two filtration procedures used are shown in Table II ($\log \bar{R}$ values) and III (plasma electrolyte concentrations and time to peak tension).

The 4 plasma fractions obtained by filtering through UM10 and UM2 filters (left panel of Fig. 1) were assayed on 230 rabbit papillary muscles (control plasma 122, shock plasma 108). Three plasma fractions were produced by the UM10 and UM05 filtrations (right panel of Fig. 1) and tested on 114 rabbit papillary muscles (control plasma 47, shock plasma 67).

All shock plasma fractions except the UM10 filtrate ($< 10\,000$ d) and the UM05 filtrate (< 900 d) differed significantly from the controls (Table II). No statistically significant difference between control and shock plasma samples was found with regard to their electrolyte concentrations (Table IV). Time to peak tension was only slightly affected by the different plasma samples (Table IV).

When comparing the effects on the papillary muscles of the different control plasma fractions with each other it was found that the UM10 filtrate ($< 10\,000$ d) differed signifi-

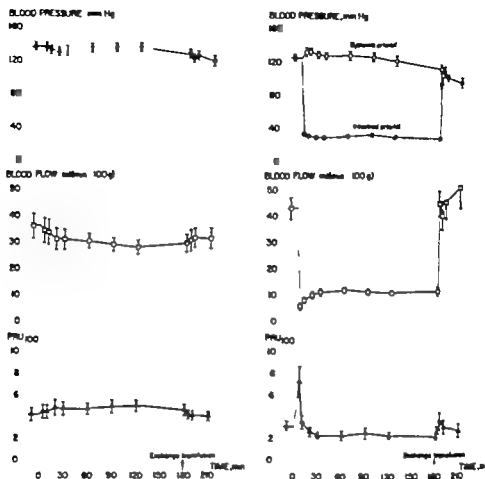


Fig. 3 Changes in arterial blood pressure, intestinal blood flow and intestinal peripheral resistance (PRU₁₀₀) in control animals (left panel $n = 13$) and in shock animals (right panel $n = 15$). Mean \pm SE.

cantly from UM05 filtrate (500–10 000 d) and UM2 filtrate (~ 1000 d), the log \bar{R} value of the UM10 filtrate being lower than that of the two other fractions. The other control plasma fraction did not otherwise differ from each other or from the original plasma sample.

The striking observation in shock plasma is that the UM05 filtrate (< 500 d) was significantly different from all other plasma samples tested (Table IV), the log \bar{R} value of this filtrate being higher than that of all the other fractions (Table II). Furthermore, the UM10 filtrate (< 10000 d) did not depress peak isometric tension as much as the original

TABLE I Microscopic grading of 1 testal control segments and of segments subjected to a 3 h period of regional intestinal hypotension. The grading was performed according to Chiu *et al.* (1970). Figures denote number of segments

	Grade of mucosal damage				
	0	1	3	4	5
Control	9	3			
Shock			1	7	3

TABLE II The experimental results obtained on rabbit papillary muscles, less exposing them to original or plasma and plasma fractions produced by ultrafiltration through Diaflo UM10, UM2 and UM05 membranes. The effects on peak isometric tension are expressed in terms of the logarithm of ratio, R , depicting the peak isometric tension developed by the papillary muscle after 15-20 min incubation divided by the peak isometric tension recorded in the control solution. Mean values \pm S.E. Numbers within parentheses denote number of plasma samples/total number of muscles assayed. S.E. values were calculated from number of plasma samples. P-values were determined with the nonparametric test of Wilcoxon. ns = not significant.

	Control plasma	Shock plasma	p-value
<i>UM10 and UM2 ultrafilters</i>			
Original plasma	0.123 ± 0.030 (8/29)	0.279 ± 0.018 (8/33)	<0.01
UM10 filtrate	-0.118 ± 0.047 (7/26)	-0.274 ± 0.041 (7/24)	0.05
UM2 filtrate	0.144 ± 0.018 (8/35)	0.226 ± 0.042 (8/30)	ns
UM2 filtrate	0.112 ± 0.019 (7/32)	-0.231 ± 0.062 (6/21)	0.05
<i>UM10 and UM05 ultrafilters</i>			
Original plasma	-0.083 ± 0.023 (5/14)	-0.234 ± 0.059 (7/14)	0.05
UM05 filtrate	-0.054 ± 0.023 (5/19)	-0.14 ± 0.013 (7/21)	0.05
UM05 filtrate	-0.054 ± 0.060 (5/14)	-0.062 ± 0.023 (7/22)	ns

TABLE III The electrolyte composition of the plasma fractions assayed on the papillary muscles and the time to peak tension measured less exposing the papillary muscles to the Krebs-Henseleit solution and to the different plasma fractions. denotes the number of plasma samples tested. Time to peak tension was in most cases determined to 4 minutes for each tested plasma sample. The total number of muscles tested are given in Table II. Mean values \pm S.E.

		Na mmol/l	K mmol/l	Ca mmol/l	Time to peak tension, msec	
					Control	Assay
<i>UM10 and UM2 filtrates</i>						
Original plasma						
Control	8	132.9±3.9	3.71±0.13	2.08±0.07	125.1±3.2	120.9±3.1
Shock	7	137.6±3.0	4.43±0.28	2.08±0.05	120.3±3.9	113.9±5.0
UM10 filtrate						
Control	7	131.0±4.9	3.70±0.21	2.14±0.21	116.6±1.6	115.3±2.6
Shock	6	137.6±4.2	4.13±0.13	2.74±0.12	116.7±2.9	112.7±5.1
UM10 filtrate						
Control	8	139.0±3.5	3.61±0.15	1.12±0.13	117.5±1.6	111.1±2.7
Shock	7	139.3±3.5	4.46±0.37	1.45±0.19	113.1±3.3	113.4±4.0
UM2 filtrate						
Control	7	152.8±13.3	3.42±0.13	1.11±0.13	116.6±2.6	113.1±1.7
Shock	5	141.7±2.9	3.98±0.29	1.21±0.10	121.6±3.6	117.7±4.2
<i>UM10 and UM05 filtrates</i>						
Original plasma						
Control	5	143.0±6.9	3.58±0.14	2.14±0.15	128.2±4.7	132.2±7.3
Shock	7	133.2±4.6	4.38±0.46	2.15±0.08	128.3±4.4	128.8±4.4
UM05 filtrate						
Control	5	151.4±5.3	3.80±0.21	2.20±0.17	129.4±4.2	130.0±4.9
Shock	7	153.0±8.2	4.29±0.31	2.81±0.07	131.0±4.2	136.4±8.4
UM05 filtrate						
Control	5	140.2±2.9	4.06±0.14	1.96±0.22	126.4±6.3	127.2±5.1
Shock	7	141.8±9.5	3.88±0.30	1.89±0.22	128.3±3.6	132.9±4.9

TABLE IV *p*-values when comparing the log \bar{R} values for the original shock plasma and the different plasma fractions produced by ultrafiltration through Diaflo[®] UM10, UM12 and UM05 membranes. The sign test and the Wilcoxon test for two samples were used if the statistical analysis is not significant.

	UM10 filtrand	UM10 filtrate	UM05 filtrand	UM12 filtrate	UM05 filtrate
Original plasma	ns	0.06	ns	ns	<0.01
UM10 filtrand		ns	<0.01	ns	<0.01
UM10 filtrate			ns	ns	0.01
UM05 filtrand				ns	0.06
UM12 filtrate					0.01

shock plasma although the numerical difference between the mean values was fairly small (Table II). The UM10 filtrand ($>10\,000$ d) exhibited a log \bar{R} value which was significantly lower than that of the UM05 filtrand (500–10 000 d).

2. *Ultrafiltration and ether extraction* In order to test if the ether extraction as such produced any cardiotoxic material the modified Krebs-Henseleit solution used in this study was extracted with ether as described in Methods. The extracted solutes were reconstituted to the original volume in the Krebs-Henseleit solution and assayed. Log \bar{R} measured -0.66 ± 0.08 (mean \pm S.E., $n=9$) in these studies, revealing only a small effect of the ether extraction procedure.

To reduce the number of muscle assays this part of the investigation was performed in the following way: plasma from 3 expts. were pooled. This solution was then filtered as described in Methods (Fig. 2) and tested on papillary muscles. Each muscle assay could in this way be considered to be statistically one observation.

The filtration procedure used in this part of the investigation produced three plasma fractions: UM10 filtrand ($>10\,000$ d), UM05 filtrand (500–10 000 d) and UM05 filtrate

TABLE V The experimental results obtained on rabbit papillary muscles when exposing them to normal and heated cat plasma and ether-extracted plasma fractions produced by ultrafiltration through Diaflo[®] UM10 and UM05 membranes. The effects on peak isometric tension are expressed as described in Table III. Mean values \pm S.E. Numbers within parentheses denote number of muscles tested (ether extractions), number of plasma samples/total number of muscles tested (heating). *p*-values were determined with the nonparametric test of Wilcoxon. ns = not significant.

	Control plasma	Shock plasma	<i>p</i> -value
<i>Ether extraction</i>			
Original plasma	-0.085 ± 0.051 (7)	-0.234 ± 0.030 (9)	0.01
Ether-extracted fraction of UM10 filtrand	-0.131 ± 0.027 (8)	-0.254 ± 0.018 (8)	<0.01
Ether-extracted fraction of UM05 filtrand	0.066 ± 0.017 (7)	0.003 ± 0.033 (9)	ns
Ether-extracted fraction of UM05 filtrate	-0.079 ± 0.022 (8)	-0.101 ± 0.011 (6)	ns
<i>Heating</i>			
Original plasma	-0.089 ± 0.019 (6/19)	-0.208 ± 0.038 (8/30)	<0.01
Heated plasma	-0.104 ± 0.020 (6/18)	-0.15 ± 0.016 (8/25)	0.05

TABLE VI The electrolyte composition of original plasma and ether-extracted plasma fractions assayed on the papillary muscles and the time to peak tension measured when exposing the papillary muscles to the Krebs-Henseleit solution and the different plasma fractions. Numbers of muscles used for estimating time to peak tension are given in Table V. Mean values \pm S.E. — number of plasma samples.

	Na mmol/l	K mmol/l	Ca mmol/l	Time to peak tension, msec	
				Control	Assay
ether extractant					
original plasma					
Control	143	3.2	1.9	127.0 \pm 9.5	134.6 \pm 10.2
Shock	136	3.4	1.8	121.9 \pm 3.9	122.8 \pm 4.6
M 10 filtrand					
Control	148	4.1	1.8	128.3 \pm 3.5	126.4 \pm 4.3
Shock	146	4.1	2.4	110.1 \pm 4.7	106.4 \pm 2.6
M 05 filtrand					
Control	150	4.3	2.2	118.9 \pm 8.6	114.3 \pm 6.9
Shock	140	4.0	2.6	117.1 \pm 4.8	112.6 \pm 4.2
M 01 filtrate					
Control	147	4.3	2.2	121.9 \pm 4.5	118.1 \pm 4.2
Shock	138	4.1	2.5	120.5 \pm 3.1	114.0 \pm 3.8
heating					
control plasma					
Original plasma (n 6)	140 \pm 4.3	3.37 \pm 0.24	1.97 \pm 0.08	129.8 \pm 4.2	133.2 \pm 6.1
Heated plasma (n 6)	139.3 \pm 4.0	3.80 \pm 0.25	2.02 \pm 0.14	124.2 \pm 4.0	121.5 \pm 4.6
shock plasma					
Original plasma (n 7)	134.3 \pm 6.6	4.47 \pm 0.41	2.12 \pm 0.07	126.9 \pm 3.8	125.0 \pm 4.6
Heated plasma (n 7)	135.0 \pm 7.3	4.99 \pm 0.34	1.74 \pm 0.15	118.4 \pm 3.4	118.7 \pm 4.6

500 d) (Fig. 2). The assay results obtained when exposing the myocardial tissue to the ether extractable material from these fractions in control and shock plasma are shown in Table V. The results from the assay on the original plasma (not ether extracted) are also shown. Comparing the control and the shock plasma samples revealed a statistically significant difference between the original plasma samples and the UM 10 filtrands (10 000 d, Table V). In both cases the shock plasma was found to cause a more pronounced depression of peak isometric tension of the papillary muscles. Comparing the different control plasma fractions with each other did not unravel any significant differences. However in shock plasma, the original plasma and the UM 10 filtrand was significantly different from the ether extracted parts of the two other plasma fractions. These fractions produced less an effect on the cardiac muscle than the original plasma and the UM 10 filtrand (Table V). The electrolyte concentrations of corresponding control and shock plasma fractions (Table VI) did not differ significantly from each other. Time to peak tension was only slightly affected by the plasma samples (Table VI).

3 **Heating** 6 control plasma and 8 shock plasma samples were heated to 55°C for 30 min. The effect of this procedure on the papillary peak isometric tension is evident from Table V. With regard to this parameter no effect of heating was observed in either control or shock plasma. Table VI presents the electrolyte concentrations and the papillary time to peak tension of the original and the heated plasma samples. No significant difference between control and shock plasma could be demonstrated with regard to these parameters.

Discussion

The molecular filtration procedures employed revealed at least 2 heat stable substances in intestinal "shock plasma" with a negative inotropic effect on myocardial tissue. One substance has apparently a molecular mass of 500-1 000 d and is insoluble in most solvents, since control and shock plasma fractions containing molecules with a mass above 500 d differed significantly (Table II), while no difference was found between the ether extracted UM05 filtrands (500-10 000 d Table V). Furthermore, among the different shock plasma fractions (Table IV) the UM05 filtrate with molecules with a mass below 500 d exhibited a significantly higher $\log \bar{R}$ value than all other plasma fractions, although the [Ca] of the UM05 filtrate was low (Table III Lundgren *et al* 1976). No such difference in $\log \bar{R}$ could be demonstrated for control plasma.

One observation in Table II is, however not consistent with the conclusion above, since $\log \bar{R}$ values of the UM10 filtrate (< 10 000 d) of control and shock plasma did not differ significantly. Numerically however the UM10 filtrate of shock plasma produced a more pronounced negative inotropic effect on the papillary muscles than control plasma. Furthermore, two other shock plasma fractions containing molecules with a mass below 10 000 d (UM2 filtrate and UM05 filtrand) depressed myocardial contractility more than the corresponding control plasma fractions. Moreover the control UM10 filtrate differed significantly from two other control plasma fractions (UM05 filtrand and UM2 filtrate) suggesting that the filtration procedure as such may have produced an effect on this particular plasma fraction.

The results reported in Table V clearly suggest that shock plasma also contained a cardiotoxic material which was possible to extract by ether at a pH of 7.5 from the highest molecular fraction in the UM10 filtrand (> 10 000 d). It seems impossible to estimate the molecular mass of this compound from the molecular filtration experiments, since the large proteins in the UM10 filtrand are transport molecules for a number of lipid-soluble substances of varying molecular mass. The existence of this cardiotoxic material in the UM10 filtrate probably explains the occasional significant difference found in the shock plasma with regard to other plasma fractions than the UM05 filtrate (Table IV).

When exposing the intestine to a period of low arterial blood pressure, it is striking that no significant blood pressure fall occurs during the intestinal arterial hypotension, while a pronounced pressure fall is seen after the regional hypotension. This observation led Haglund and Lundgren (1974) to propose that part of the cardiotoxic material produced in the hypoperfused gut was fairly lipophilic and of comparatively small molecular mass so as to be easily trapped in the intestinal countercurrent exchanger during the period of hypoperfusion, which in turn, delays its net blood absorption. The presence of a lipophilic cardiotoxic material was further strengthened by the observations of the present study discussed above.

To summarize, the present study suggests that the feline small intestine in hypotension produces at least two heat stable compounds with negative inotropic effects on the myocardium. One is water soluble with a molecular mass of 500-1 000 d and another lipophilic, possibly also of small molecular mass.

The presence of toxic factors in various types of shock has been proposed by a number of investigators as reviewed by Gruber (1967) and Lefer (1973). In the latter review eight different "factors" were listed, all of which seem to be formed in the splanchnic region. Most of these are of large molecular mass (above 10 000 d) and the only heat stable water soluble "factor" of small molecular mass, with cardiotoxic effects resembling the one of the present study is the myocardial depressant factor (MDF) described by Lefer (1970, 1974). MDF is believed to be a peptide or a glucopeptide having a molecular mass of 800-1 000 d, it is heat stable and soluble in water but not in the nonpolar solvent methylene chloride. In all these respects MDF resembles the hydrophilic cardiotoxic material of the present study. A number of experimental observations suggest, however, that the two cardiotoxic materials may not be chemically identical.

First, MDF is according to Lefer and co-workers mainly released from the ischemic pancreas. In the present shock model a large portion of the pancreas was extirpated and the remaining part was perfused at normal arterial pressure. Second, Glenn and Lefer (1971) were unable to demonstrate the production of MDF by the duodenum *in vitro* although MDF was produced under similar conditions in pancreatic tissue. Third, the myocardial depressant effect of the present study was caused by material in the intestinal venous blood while MDF is transported from the pancreas via the lymphatics. Evidence for this was presented by Glenn and Lefer (1970) who showed that thoracic lymph diversion prevented the appearance of MDF in plasma in cats subjected to hemorrhagic shock. Fourth, Lefer and Martin (1969) filtered dialyzed shock plasma through a UM2 membrane cut off 1 000 d and found depressant activity in the filtrate but none in the filtrand. In the present study cardiotoxic material was found in plasma fractions containing solutes with a molecular mass below as well as above 1 000 d (Table II). In fact, if MDF is a molecule with mass below 1 000 d it should have appeared both in the filtrand and in the filtrate of the UM2 membrane. Thus, although the chemical properties of MDF and the water soluble cardiotoxic material of this study exhibit similarities, they may not be chemically identical.

In the review by Lefer (1973), one of the enumerated "factors" is soluble in nonpolar solvents, i.e. the retractor endothelial-depressant substance (RES) described by Blattberg and Levy (1962). This material was soluble in methylene chloride and it also exhibited some cardiotoxic activity (Lefer and Blattberg 1968). However, RES was possible to extract with methylene chloride from a protein free solution (Blattberg and Levy (1966)), while the hydrophilic material of this study was found only in the transport protein fraction of plasma.

Fine (1967) has proposed that bacterial endotoxins absorbed from the intestine during shock are one important contributing factor to the development of so called irreversible shock. No determinations of endotoxins were performed in this study. However, endotoxins have been shown not to depress myocardial contractility (Hilleshaw *et al.* 1973). Furthermore, endotoxins are water soluble large molecules with mass between 200 000 and 1 000 000 d. Hence, it seems highly improbable that they are chemically identical to the cardiotoxic material reported in this study.

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Influence of local noxious heat stimulation on sensory nerve activity in the feline dental pulp

By

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Abstract

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The present investigation is undertaken to develop an experimental model in which noxious heat stimulation was used to produce increased intradental sensory nerve activity in catlike teeth of anesthetized cats. Two techniques are evaluated in which both the method of recording and the nature of the stimulus varied. Slow heating (approx. $1^{\circ}\text{C}/\text{s}$) to 47°C of the tooth surface (combined with recording from electrodes in open distal cavities) did not produce any persistent nerve activity. Repeated periods of brief extreme heating (approx. $60^{\circ}\text{C}/\text{s}$) (combined with recording from amalgam electrodes placed on cavity floors) resulted in an immediate response and an afterdischarge (phase 3) generally persisting for 20-60 min. Maximum phase 3 activity as characteristics for the individual cat and ranged from 0.2 to 50.2 $\mu\text{V}/\text{s}$, mean value 10.6 $\mu\text{V}/\text{s}$ (S.D. = 9.2). A systematically higher phase 3 activity was recorded in lower compared to upper canine teeth ($p < 0.05$). The maximum phase 3 response generally occurred after 3-8 stimulations, the median number of required stimuli was 3. Repeated brief heat stimulations combined with the closed cavity recording technique may be used as an experimental model by which the mechanisms behind increases in intradental sensory nerve activity associated with tooth damage can be studied.

Injury to a tooth produced by thermal, chemical, bacterial or mechanical insults can result in the clinically observed phenomenon of acute pulpitis with hypersensitivity to stimuli and/or severe pain. Little is known of the mechanisms by which these effects are produced. The purpose of the present study was to develop an experimental model in which thermal stimuli applied locally to the teeth of anesthetized cats could be used to produce similar effects, the degree of which could be assessed by recording the associated intradental sensory nerve activity. Such a model could be used to study the mechanism of acute pulp injury and the effect of interventions which may modify its expression.

Material and methods

The experiments were carried out on adult cats (2-4 kg). The animals were anesthetized with sodium pentobarbital (30 mg/kg) or chloralose (40 mg/kg) with urethane (30 mg/kg). Immobilization, cavity preparation and experimental procedures were performed as described by Olsson, Hagerstrom and Edvall (1974). Two techniques were evaluated in which both the method of recording and the nature of the thermal stimulus were varied.

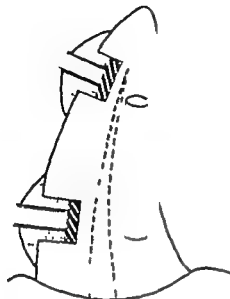


Fig. 1 Drawing showing electrode contacts implanted intradentally and the tube for the thermistor fixed to the root surface.

Open cavity recording—slow heating of tooth

Two cavities were prepared in the canine tooth, one over the pulp horn, the other within the gingival furrow of the crown. The cavities were filled with isotonic saline solution and surrounded with Plast base (Squibb). Recording electrodes were placed in the cavities and recordings were obtained from intradental sensory nerves (for details see Olgart 1974). The responsiveness of the preparation was tested by the application of 9% sodium chloride solution to the coronal cavity. If no clearcut neural activity was recorded within 1 min after applying the hypertonic solution to the exposed dentin, the cavity was deepened until such response was obtained. Washing the cavity with isotonic saline solution rapidly abolished the activity. Tooth temperature, monitored by a thermistor placed at the lingual surface, was maintained during control period at 34°C by a water circulated thermode placed in contact with the tooth crown. Cold stimulation was produced by decreasing the thermode temperature to 20–27°C. The thermal injury in this series of experiments was attempted by raising the thermode temperature by approximately 1°C/s to a maximum of 46–47°C. Stimulation was generally applied for 10 min. After stimulation the temperature at the tooth surface returned to resting level at a rate of about 1°C/s.

Closed cavity recording—brief intracavity heating of tooth

A modified technique developed by Edwall and Olgart (1977) was used to record intradental neural activity (Fig. 1). Two cavities were prepared and deepened to about half the distance from enamel to pulp. After partial drying, the floor of each cavity was covered with a layer of amalgam in which stainless steel tubes (\varnothing 0.3 mm, length about 2 mm) were secured by composite filling material (Concise). Connecting leads were inserted into the tubes and the signals obtained were amplified and displayed on a cathode ray oscilloscope using conventional techniques and stored on magnetic tape (Edwall and Scott 1971; Olgart *et al.* 1978). The signals were counted on a scaler using pulse amplitude discrimination and mean impulse frequency expressed as imp/s, was printed out for successive 10 periods. The form and amplitude of recorded signals did not seem to alter during an experimental session using either recording techniques. Another tube (\varnothing 1.2 mm length about 4 mm) containing a thermistor was fixed to the tooth surface to register temperature at that site. Responsiveness of this preparation was tested by a cold stimulus produced by application of a cold pellet soaked with ethyl chloride to the mesial part of the tooth surface for 2–10 s. This caused the surface temperature of the tooth, measured by the thermistor at half the crown length (resting temperature about 30°C), to decrease 6–10°C. Thermal injury as produced by the application to the tip of the tooth for up to 1 min of gutta-percha (De Trey's Provisorische Gutta-percha) heated in a flame. This increased the temperature at the surface thermistor to 37–38°C. Experiments in which a thermistor was placed at the tip of the tooth surface, revealed that at the site of application of the gutta-percha, the temperature rose to 85–90°C in less than 1 s. Immediately after stimulation the temperature decreased and returned to resting level in 4–5 s. In 3 experiments, which pulp temperature was recorded, the temperature

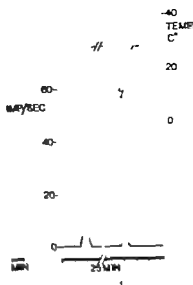


Fig. 2. Influence of cold stimulation on impulse frequency in adult 3.0 kg. Pentobarbital. Closed cavity recording. Temperature (—) impulse frequency (---). Cold stimulus, ethyl chloride.

induced into the pulp here, it rose to a maximum of 44–48°C within 2 sec and returned to resting level in about 15 sec after removal of the gateperches. The corresponding increase in temperature in the middle part of the crown pulp was 2°C (resting pulp temperature 32.5).

To evaluate the long-term effect of repeated brief heat stimulation on the neural activity and on the morphology of the pulp, series of chronic experiments were performed (6 teeth, 3 cats). After the first experiment each animal was allowed to recover from the anaesthetic. The procedure was repeated 3–4 times at weekly intervals and following the last experimental session the teeth, which had been heated, were extracted and the pulp was removed by conventional histological technique. Two teeth, prepared in the same way but exposed only to repeated cold stimulation served as controls for the histological studies.

Results

Open cavity recording

6 experiments were conducted in 3 cats in study this model. In general no spontaneous activity was recorded, and slow cooling produced only occasional single impulses even on repeated stimulation. Heat stimulation for about 10 min induced a few single impulses or a transient low frequency activity starting when the surface temperature of the tooth had reached 46°C. No activity persisted after stimulation. Repeated and prolonged stimulation (max 30 min) did not provoke any increase in the activity but resulted in a loss of responsiveness to hypertonic saline solution (3 teeth). In these cases exploratory pulp exposure did not produce bleeding which suggested that pulp necrosis had occurred. A thermistor inserted into the pulp revealed pulp temperature of 46°C when the surface temperature of the tooth had been kept at 47°C for 12 min.

Closed cavity recording

No initial spontaneous activity was recorded with the exception of some single impulses immediately after preparation. The cold stimulus resulted in a burst of recorded electrical

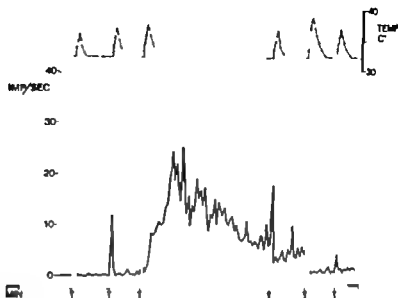


Fig. 3 Influence of repeated heat stimulation on impulse frequency. Temperature (---). Impulse frequency (—). Cat, adult 2.1 kg. Chloralose-urethane. Closed cavity recording. 1 Heat stimulation, temper guttapercha.

activity which was closely related to the fall in temperature at the tooth surface and was reproducible (Fig. 2). Brief application of heated guttapercha to the tip of the tooth elicited a burst of impulses generally lasting about 10 s. Initially there was a prompt fall of electro activity to baseline level, but if the stimulus was repeated this prompt response was succeeded by a longer lasting increase in neural activity which reached a maximum 2–4 min after the stimulus, but was detectable for 20–60 min. This afterdischarge generally appeared after the 2nd stimulus and increased with further stimuli the largest effect being observed after the 3rd to 8th application in different expts. (Fig. 3). Continued application of heat stimulation led to diminution and finally loss of all responses including that associated with cold stimuli.

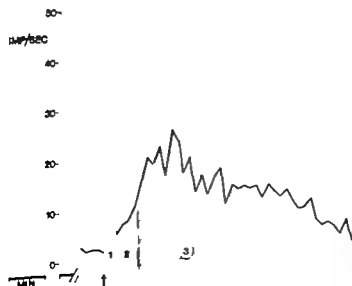
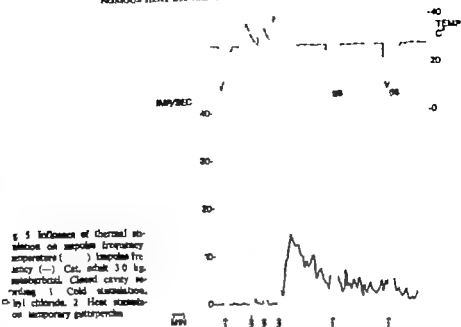


Fig. 4. Impulse frequency response induced by repeated heat stimulation. Closed cavity recording. Arrow indicates third heat stimulation. Phase 1 2 10 s. 2. Phase 2, 3 10 s. 3. Phase 3 18 10 s.



erion. The prompt response to heat and the more prolonged activity were often separated by a short period when recorded activity approached or reached the baseline level. The complete response can therefore be divided for descriptive purposes into 3 phases, an immediate response (phase 1), a variable latent period (phase 2) and a prolonged afterdischarge (phase 3)

TABLE I. Maximum phase 3 activity as impulses/second, induced by repeated heat stimulation. C+ denotes the right upper and C- the left lower cusp

Animal no	Maximum phase 3 activity imp/s				Mean imp/s
	C	C	C	C	
1	11.1		16.2		14
2		6.9		11.4	7.7
3		6.9		3.3	4.9
4	2		2.0		2.1
5	1.1	0.9	0.9	1.2	1.0
6		7.8	8.5		8.2
7	17.6			20.8	19.2
8	1.4	2.7	2.3	3.2	2.4
9	4.9			5.5	5
10	7.0		9.7		8.4
11	35.2			30.2	42.7
12	9.5			15.1	12.5
13	2.1			0.6	1.4
14	7.3			15.7	11.5
15		8.1	14.4		11.3
16		24.6		28.5	26.6
Number of teeth	C	C	C	C	Total
Means of maximum phase 3 activities	8.7		11.5		10.0



Fig. 6. Section of tooth which has been stimulated with heat on 4 experimental occasions. Note the presence of odontoblasts and the extensive tissue alterations in the initial part of the pulp. The nec cavity is seen in the upper right corner

(Fig. 4) A quantitative expression of the phase 3 activity was derived by calculating the mean number of impulses per second over the period from 1–4 min inclusive after stimulation *i.e.* excluding the first and second phases of the response. If the cold stimulus was applied during a period of phase 3 activity induced by heat, a markedly increased response was obtained compared to control responses (Fig. 5)

The maximum phase 3 activity recorded from 48 teeth in 27 cats and expressed by the above mentioned parameter showed considerable variation between the individual animals and ranged from 0.2 to 50.2 imp/s with a mean value of 10.6 imp/s ($S.D. \pm 9.2$). The median number of required stimuli was 3. Far less variation in maximum phase 3 activity was

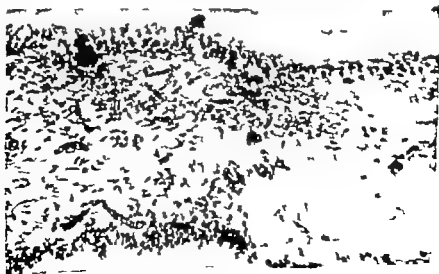


Fig. 7. Same pulp, higher magnification. Demarcation zone showing tissue undergoing necrosis.

and within the individual animals. Table I shows the maximum phase 3 activities obtained in 16 of the cats, in which heat stimulations were applied to one upper and one lower incisor tooth or to all four canine teeth. A systematically higher phase 3 activity was recorded in lower compared to upper canine teeth ($p < 0.05$ sign test).

In chronic expts. in which animals were studied at weekly intervals, a gradual diminution of the responses was observed and on the 3rd and 4th experimental sessions little or no phase 3 activity could be recorded. The responses to cold and phase 1 response to heat were considerably reduced but still present. Histological examination of these teeth showed severe morphological alterations in the incisal part of the pulp: the odontoblasts were markedly reduced or absent, the structure of the pulp tissue was changed and the number of cells was reduced. In one tooth also a demarcation zone, showing tissue undergoing necrosis was seen in the incisal part of the pulp (Fig. 6 and 7). In control teeth to which only cold stimulation was applied the responses to the stimulus derived at the 3rd or 4th experimental occasion were of about the same magnitude as recorded in the initial expt. These teeth showed only minor morphological changes in the pulp tissue.

Discussion

Thermal stimulation of teeth in cats and dogs has previously been shown to evoke activity in dental nerves (Piaffano 1939, Wagers and Smith 1960, Furukoshi and Zotterman 1963, Yamada, Sazuta and Higuchi 1963, Matthews 1967, 1968, 1977), but little information is available about sensitization of intradental nerves following heat stimulation.

Heat stimulations have been found to result in sensitization of nociceptors in skin of cat and monkey (Beck, Handwerker and Zimmermann 1974, Betzel and Dubner 1976, Croze, Duchaux and Kenshalo 1976) and in skeletal muscle of dog (Kumazawa and Mizumura 1977), characterized by a decrease in threshold temperature and an increased frequency of discharge during stimulation. A low-frequency afterdischarge lasting for some minutes was reported by Betzel and Dubner (1976) following long duration of the stimulus (30 s) or large temperature shifts into the noxious heat range. They also found that intense noxious heat depressed the response, which they ascribed to heat damage of the receptor. An after discharge was frequently observed especially on repeatedly heated cases (Kumazawa and Mizumura 1977).

In the present study brief application of heat to the tooth combined with a closed cavity recording technique was found to be an effective stimulus to obtain a persisting intradental nerve activity. Recent studies have shown that the nervous activity recorded by the present techniques originates from intradental sensory nerves (Arwill *et al.* 1973, Haegerstrom 1976) and that it correlates with the sensation of pain in man (Edwall and Olsson 1977). Brief heat stimulation resulted in a complex pattern of neural activity in which 3 phases can be distinguished. The immediate response (phase 1) was probably due to thermal gradients inducing hydrodynamic excitation of the sensory nerves (Brinmstrom and Astrom 1972). Phase 2 might be interpreted as a period of latency in which the temperature returns to normal and pathological alterations develop in the injured pulp. The activity during phase 3 is more interesting since it occurred after the stimulus had been removed. This suggests

that phase 3 activity must be due to some persistent damage to the pulp. Support Interpretation is obtained from a study on monkey by Zach and Cohen (1966). They that heat stimulation for 5-20 s, inducing an increase in pulp temperature of about 6°C resulted in morphological changes such as destruction of the odontoblasts, cell aspiration, hyperemia, edema and, in 15% of the teeth, pulp necrosis. After an increase of about 11°C abscess formation was seen in 60% of the teeth. In the present study a temperature of 44°C was recorded at the pulp horn, indicating that localized inflammatory changes in the pulp tissue could be expected and evidence of tissue damage was obtained from the histological examination of pulps which had been subjected to repeated heat stimulation in chronic experiments. The finding that repeated heat stimulation was required to produce maximum phase 3 activity indicates that the effects were additive, gradually increasing the severity of the pulp damage. This suggestion is supported by the observation that further heat stimulation led to diminution of the neural responses in acute as well as in chronic experiments. Depressed responses following repeated noxious heat stimulation has previously been observed in pulpal sensibility (Pfaffmann 1939 Wagers and Smith 1960).

The maximum phase 3 activity was found to be characteristic of the individual cat and in particular cat the lower canine teeth generally gave a higher activity than the upper canines. A quantitative expression of maximum phase 3 activity seems to offer a useful parameter in comparative studies on intradental sensory nerve activity especially in homologous paired teeth. The variability in maximum phase 3 activity seen within such pairs is not surprising since the number of activated sensory units available for recording may differ from one preparation to another and the heat stimulations were only roughly standardized. If this parameter should be used for experimental evaluation of the sensation of pain as a model implication of pulp injury it has to be regarded that the present recording techniques only permit recording of peripheral neural activity within the coronal part of the pulp and from only some types of nerve fibres (Haegerstrom 1976).

Slow heat stimulation of the tooth in combination with the open cavity recording technique was found to be an unsatisfactory model for studies on increased intradental nerve activity despite evident damage to the pulp indicated by complete loss of reactivity and necrosis. It seems unlikely that the difference in recording technique (closed or open cavities) could explain the failure to evoke neural activity by slow heat stimulation since Edwall and Ohlsson (1977) reported similar responses using either technique. Another possibility is that the technique to apply the heat stimulus can account for the different results.

Brännström and Åström (1972) proposed that pain elicited by thermal stimuli is produced by fluid movements produced thereby in the dentinal tubules. The poor responses to heat stimulation in the open cavity experiments suggests that raising the temperature at a rate of 1°C/s is inadequate to provoke such hydrodynamic excitation of the intradental sensory nerves. Furthermore repeated and prolonged heat stimulation caused the loss of responsiveness and impairment of blood supply suggesting that considerable tissue injury had taken place. One explanation could be that the uniform heating of the pulp produced by this method leads to widespread functional impairment of tissues including nerves so that the pulp tissue located between the electrodes from which the recorded electrical activity originates (Haegerstrom 1976) is incapable of generating observable impulses. The pulp has been

by Póto and Scheinin (1958) to be extremely vulnerable to heating, a period of 2 min at 45°C being sufficient to lead to arrest of blood flow with stasis and thrombosis ensuing. Such changes could be expected to reduce neural reactivity (Edwall and Scott 1971) and could be occurring at temperatures close to the expected threshold for impulse generation (Funakoshi and Zotterman 1963). Therefore it cannot be excluded that nerve endings stimulated further apically might have been excited though recordings cannot be obtained by present techniques. Support for this view can be found in studies where nocuous heat stimulation was applied to teeth and neural activity could be recorded from pulpal fibres in the alveolar nerve (Pfaffmann 1939, Wagers and Smith 1960, Funakoshi and Zotterman 1963, Matthews 1967, 1968, 1977).

The increased neural activity and excitability produced in the tooth in this study may well be associated with the formation of inflammatory mediators which are known to affect nerves. If this proves to be the case, the parameters discussed above could be used to evaluate the effect of other influences modifying the inflammatory process. Repeated brief heat stimulation in combination with the closed cavity recording technique has already been used to examine the action of anti-inflammatory drugs on the responses of pulp tissue to thermal injury (Höberg 1978).

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Reinnervation of partially denervated rat soleus muscle

By

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Abstract

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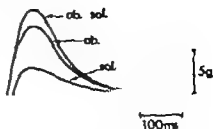
The reinnervation of partially denervated rat soleus muscles by their interrupted, regenerating motor axons has been examined in adult rats. If reinnervation occurred after the regrowing, intact motor axons had sprouted to their full axonal extent, then the regenerating axons formed synapses preferentially with denervated muscle fibers and not with fibers innervated by sprouts. The sprouted motor units retained their size as if no reinnervation had occurred. On the other hand, if reinnervation occurred early during the sprouting process, the sprouting motor units were never able to attain their maximal size. Further, some muscle fibers became innervated by both sprouted and regenerating axons. These "hyperinnervated" fibers lost their dual innervation after a few weeks. The sprouted axons seemed to be the nerve fibers preferentially eliminated from these hyperinnervated fibers, since during the loss of hyperinnervation the sprouted motor units decreased in size while the motor units formed by the regenerating axons did not change in size. It is proposed that the occurrence of hyperinnervation is followed by the amount of time sprouting axons have to consolidate their synapses with muscle fibers. Further, it is proposed that one muscle fiber which has become hyperinnervated, the sprouted motor axons are at disadvantage in the competition for transmission because of their larger unit sizes.

The number of synapses which a motor neuron forms within its target muscle is limited. In the soleus muscle of the adult rat this limitation is set by a constant or nearly constant number of muscle fibers, a narrowly defined number of motor neurons, and a requirement that each muscle fiber receive innervation from only one neuron. A result of these limitations and an incompletely understood post-natal development (Redfern 1970, Brown *et al.* 1976) is that the soleus motor neurons divide the soleus muscle fibers among themselves so that each neuron innervates about the same number of muscle fibers, *i.e.* the motor units are of roughly uniform size.

Adult soleus motor neurons can, however, be induced to increase the number of muscle fibers they innervate if the muscle is partially denervated, *i.e.* if the axons of a portion of the population of the motor neurons are interrupted (Cluett 1962, Thompson and Jansen 1977). This expansion in motor unit size constitutes the well-known phenomenon of collateral sprouting which has been shown to occur in many central and peripheral neurons in response to partial denervation (Edds 1953, Ransman and Field 1973). In the case of the adult soleus

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Fig. 1. Summation of twitches from the sprouted nerve and the reinnervated nerve in a muscle partially denervated by sciatic nerve cut 133 days earlier. Superimposed traces of the twitch to stimulation of the sciatic nerve alone (sol), to the aberrant nerve alone (ab) and to stimulation of both nerves simultaneously (ab + sol). The timing of the stimulus was adjusted so as to give the maximal twitch. The aberrant nerve in this muscle contained 3 motor units.



Muscles partially denervated by nerve cuts

When partial denervation was achieved by cutting the soleus nerve, reinnervation of the soleus muscle was first observed at day 12 after partial denervation. Six rats were examined 16 to 133 days after such partial denervation in order to determine the extent of hyperinnervation and the sizes of motor units.

The degree of hyperinnervation in these muscles was determined by two procedures. First, the summation of twitch tensions to the aberrant and the regenerated soleus nerve was measured. A typical result of this type of measurement is illustrated in Fig. 1. Here it can be seen that the tension generated by the two nerves stimulated together is approximately equal to the sum of the tensions when they were stimulated separately. Such a result was the case for all 6 muscles examined (Table I), and this result suggests that the aberrant nerve and the soleus nerve innervate different populations of muscle fibers in the muscle.

A more accurate way to estimate the degree of hyperinnervation in these muscles is to sample the innervation of individual muscle fibers by microelectrode recording. This method also has the advantage that it will reveal the existence of any innervation which might be too weak to effect contraction in the muscle. The end-plate potentials (epps) from the sprouting and regenerating axons were found only near the center of each muscle fiber at the site of the old denervated end-plates. With a few exceptions, muscle fibers gave epps only to one of the nerves (cf. Table I). Only 2 of 92 fibers in 5 muscles had epps to stimulation of both the aberrant and the soleus nerves. Hence, both tension measurements and intracellular recordings indicate that the regenerated and the sprouted nerves innervated, with rare excep-

TABLE I. Hyperinnervation of muscles partially denervated by nerve cut.

Age (days)	Tension measurements				Epp recordings				
	Aberrant N.		Soleus N.		Ab N. Sol. N.	convergence	No. of fibers with both epps.	No. of these also ab epps.	convergence
	Tension (g)	Number of units	Tension (g)	Number of units					
18	7.1	ca 10	3.1	10	7.7	6.5	30	1	3.3
22	7.6	5	0.6	3	7.9	3.8	18	1	5.6
12	16.6	11	1.3	3	17.6	2.9	12	0	0
17	8.7	3	1.2	4	9.2	7.6	12	0	0
49	12.2	6	2.1	7	19.1	6.3	—	—	—
111	7.5	3	2.7	10	10.0	2.0	26	0	0

muscle partial denervation causes motor units to increase their size to on the average, about 3-4 times their normal size (Thompson and Jansen 1977). This increase in size seems to persist indefinitely at least as long as the axons interrupted upon partial denervation do not reinnervate the muscle.

Guth (1962) has reported that when interrupted axons return to partially denervated rat soleus or plantaris muscles many of the muscle fibers already innervated by the sprouted motor neurons also came to be innervated ("hyperinnervated") by the regenerating axons. This observation is interesting since it means that muscle fibers which are innervated are active accept additional innervation. Normally such muscle fibers are refractory to hyperinnervation (Eisberg 1917).

I have reexamined the problem of reinnervation of partially denervated rat soleus muscle and confirm that hyperinnervation does occur although to a limited extent. Furthermore, the hyperinnervation is transient. Many of the muscle fibers lose their innervation from the sprouted nerve and remain innervated only by the regenerating nerve. Independently Brown and Irons (1977) and Betz (1977) have obtained similar results from other muscles.

Materials and Methods

Animals used in these experiments were a special strain of albino rats (AO strain, MRC Cellular Immunology Unit, Oxford) whose soleus muscles possess a unusual pattern of innervation (Thompson and Jansen 1977). In addition to the normal soleus nerve which is a branch of the tibial nerve, the soleus muscle of these rats frequently receive innervation via a second, smaller nerve, the so-called aberrant nerve which arises as a branch of the plantar nerve. The earlier experiments have established that this aberrant nerve possesses a variable number of the normal complement of soleus motor axons which reach the muscle via this unusual path instead of through the soleus nerve.

Soleus muscles in these animals were partially denervated by either resecting, cutting, or crushing the lateral gastrocnemius-soleus nerve at the point where it branches from the tibial nerve in the thigh. These operations were conducted under ether anesthesia. The separate methods of partially denervating the muscles were selected because they provide for different nerve regeneration times (Frank *et al.* 1975). In the first method the nerve was cut with a pair of dissecting scissors. In the second method the nerve was crushed several times between the smooth tips of Dumont number 3 wickmaker's forceps. The third method consisted of resecting a 1 mm piece of the nerve and ligating the proximal stump with 9-0 surgical thread. Wounds were sutured closed and a local anesthetic and penicillin (150 000 units/kg, i.m.) administered.

For the acute experiments, the muscles and their nerves were dissected under a microscope in oxygenated Ringer's solution (composition in mM: NaCl, 137; KCl, 5; CaCl_2 , 10; MgCl_2 , 1; glucose 10; TrisCl , 10, buffered to pH 7.4). After dissection the muscle/nerve preparation was removed to a chamber through which oxygenated Ringer's was perfused (rate ca. 1 ml/min). Isometric tetanizations were measured by attaching the distal tendon by 6-0 silk suture to a sensitive strain gauge (0.15 V/g). The length of the muscle was adjusted to obtain the maximum twitch tension. Nerves were stimulated through suction electrodes. Muscles were directly stimulated by 1 msec duration pulses delivered through silver electrodes on each side of each muscle. Evoked synaptic potentials were measured in curarized muscles (2-6 10^{-6} g/ml D-tubocurarine) in intracellular microelectrodes (20-50 M Ω , potassium acetate filled).

After physiological examination, most of the partially denervated muscles were prepared for histology by fixation in 2% paraformaldehyde-2.5% glutaraldehyde and post fixation in osmium tetroxide. The muscles were embedded in plastic and 3 μ thick cross-sections were made through the central region and stained with toluidine blue. The number of muscle fibers in the muscle cross-sections was counted on photographic montages.

Results

The result of the reinnervation of partially denervated soleus muscles differed according to the time of arrival at the muscle of the regenerating motor axons.

TABLE II. Hypertennervation of muscles partially denervated by nerve crush.

Days	Tension measurements				Action potentials				End-plate potentials			
	Ab Nerve		Sol Nerve		Ab + Sol tension (g)	% con- vergence	Sol N	Sol & Ab	% con- vergence	Sol N	Sol N + Ab N	% con- vergence
	Tension (g)	No. of units	Tension (g)	No. of units								
0	5.9	9	1.9	>14	6.7	16	—	—	—	18	5	28
9	4.3	5	3.4	19	6.6	17	20	3	15	18	3	17
10	2.6	2	3.8	>19	7.1	18	52	6	19	20	5	11
10	4.2	7	2.4	18	3.4	22	11	2	18	28	2	7
17	8.2	6	12.1	20	17.5	16	—	—	—	27	0	11
28	1.4	1	11.2	26	12.9	5	21	1	5	—	—	—
30	4.6	—	17.9	20	20.0	12	20	1	5	18	0	9
38	12.1	8	10.7	19	18.6	23	—	—	—	33	1	3
46	8.5	6	12.5	>20	16.8	24	47	0	8	31	0	0
51	6.4	4	11.4	>20	15.4	16	26	1	4	10	0	0

the muscle. In addition to the procedure shown in Fig. 2, the muscle was also stimulated directly by currents passed between two electrodes on each side of the muscle in order to determine the maximal twitch the muscle was capable of generating. The ratio of each increment in twitch tension to the maximal direct tension is taken to be a measure of the fraction of the muscle fibers in the muscle innervated by each motor axon. Each fraction was then converted into a number of muscle fibers by multiplying by the number of muscle fibers counted in a cross-section of the muscle.

The results of these unit size measurements for the muscles partially denervated by lateral gastrocnemius-solens nerve cut are shown in the histogram of Fig. 3. The units from the 6 muscles had an average unit size of 517 fibers. For comparison, the results are also shown from 3 muscles in which there was no solens nerve reinnervation 19-67 days after partial denervation (reinnervation was prevented in these muscles by resecting a portion of the nerve and ligating the proximal stump). Clearly there is no discernible difference in the unit size. Hence, the sprouted aberrant motor units are not appreciably decreased in their expanded size upon reinnervation by the solens nerve. The regenerating axons seem to have preferentially innervated muscle fibers which had not been claimed by the sprouting axons.

Muscles partially denervated by nerve crushes

In second group of rats partial denervation of the solens was achieved by crushing the lateral gastrocnemius-solens nerve with forceps. This procedure allowed for more rapid reinnervation by the interrupted solens nerve axons. Strong reinnervation was noted at 9 and 111 days post-partial denervation in 4 of these animals, weak reinnervation was seen in one rat examined at 7 days. Twitch tensions were measured in these muscles and once again the degree of convergence was only moderate. The values are given in Table II, and they were between 5 and 15%.

However, when action potentials and end-plate potentials were recorded in these muscles, a considerable hypertennervation was detected at early times. In 4 crushed muscles examined

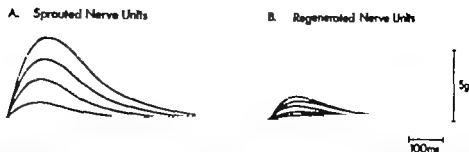


Fig. 2. Twitches of sprouted (aberrant) nerve motor units (part A) and regenerated (soleus) nerve motor units (part B). The muscle was partially denervated by crush of the soleus nerve 71 days earlier. The aberrant and the soleus nerve were in turn stimulated with shocks of carefully graded intensity while measuring isometric tension generated by the muscle. Each all-or-nothing increment in the twitch tension produced by the activation of a motor axon in the nerve was recorded on the face of a storage oscilloscope. The stimulus intensity was adjusted several times to ensure reproducibility of the responses. Part A shows the 4 units of the aberrant nerve. Part B shows the 5 units of lowest threshold in the soleus nerve.

tions, separate fibers in these muscles. It should also be noted that the two doubly innervated fibers were found in the animals with the shortest survival times.

To determine whether reinnervation changed the sizes of the sprouted motor units, we examined the motor unit sizes of aberrant motor axons in these partially denervated muscles with soleus reinnervation and compared them with the aberrant motor unit sizes in partially denervated muscles without soleus reinnervation. Motor unit sizes were determined using the procedure described previously (Thompson and Jansen 1977). Fig. 2 provides an illustration of this procedure for an adult muscle partially denervated 71 days previously. Fig. 2 A shows the tension increments for the 4 units present in the aberrant nerve. 2 B shows the tension increments for the 5 units of lowest threshold in the soleus nerve which had regenerated to

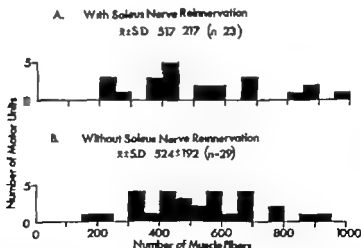


Fig. 3. Sprouted nerve motor unit sizes with (A) and without (B) soleus nerve reinnervation. Part A shows 23 aberrant nerve motor units obtained from 6 muscles 16-133 days after partial denervation by soleus nerve cut. In each of these 6 muscles, there was extensive soleus nerve reinnervation. Part B shows 29 aberrant nerve motor units obtained from 8 muscles 19-67 days after partial denervation. In these muscles partial denervation was accomplished by resection and ligation of the soleus nerve which did not reinnervate the muscle.

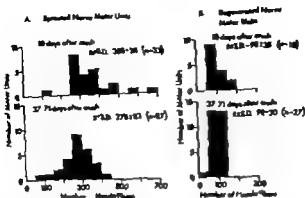


Fig. 5. Sizes of sprosted and regenerated motor units in muscles partially denervated by crush of the soleus vs. The data are divided into two histograms according to survival time after nerve crush: top histograms both A & B give the motor unit sizes for 4 muscles examined 9-10 days after nerve crush; the lower histograms give the motor unit sizes for 4 muscles examined 37-71 days after nerve crush. Motor unit sizes (the data presented here were computed using 3,400 fibers for the normal motor present in each muscle). muscle fiber counts made of some of the muscles, an average of 3,340 was obtained.

in muscles where reinnervation by the soleus nerve was either prevented or delayed about a week longer (averages of 574 and 517 fibers, respectively see Fig. 3). Apparently if sprouting nerve fibers reach the muscle soon after partial denervation they are able to compete with the sprouting nerve fibers for innervation of the available, denervated muscle fibers. However the regenerating soleus nerve motor units were never able to regain their normal size. The soleus motor units after regeneration through the crush (8 ± 3 , D = 94 ± 32 , Fig. 5 B) are significantly smaller (Wilcoxon, $P < 0.001$) than the normal soleus motor units (150, Thompson and Jansen 1977).

Further measurements of the sizes of the sprosted motor units indicate that these units may be decreasing in size with time after partial denervation. The sprosted motor units are larger at 9-10 days after partial denervation than they are at later times (37-71 days). This difference is statistically significant (Wilcoxon, $p < 0.01$), and suggests that some of the sprouting axons which have been fully functional lose in the competitive interactions with the regenerating axons. Presumably this applies to the muscle fibers which were doubly innervated at early times. This inference is further supported by the finding, shown in Fig. 5 B that the motor units of the regenerating nerve do not significantly change in size.

During the course of these experiments, a potential complication in this type of experiment was discovered which could severely distort the interpretation of the results. In a number of experiments stimulation of the soleus nerve close to the muscle (distances of 2-5 mm from the muscle) gave a vigorous contraction of the muscle whereas more distant nerve stimulation gave no response. The responses to close stimulation were clearly not due to stimulus spread to the aberrant nerve, as they were abolished once the soleus nerve was removed from the stimulating section electrode. Further characteristics of this type of soleus nerve response are illustrated in Fig. 6. As shown in part A, the soleus nerve evoked a twitch of amplitude and rise time equal to that of the aberrant nerve twitch. Further when the soleus nerve and the aberrant nerve were stimulated together the muscle gave a twitch response which was no

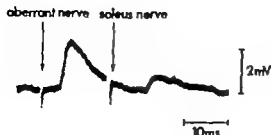


Fig. 4 Intracellular recording from a muscle fiber innervated by both the sprouted (aberrant) nerve and the regenerated (soleus) nerve. The fiber is a muscle partially denervated by soleus nerve on 9 days earlier D-tubocurarine $2 \cdot 10^{-4}$ g/ml.

9 and 10 days after partial denervation from 7 to 28% of the muscle fibers which gave an epp to the aberrant nerve also gave an epp to stimulation of the regenerated soleus nerve (Table II). An example of one such recording can be seen in Fig. 4. In contrast only one such doubly innervated fiber was found in muscles examined after long survival times. Comparable results were obtained by examining the frequency of action potentials elicited by stimulation of the two nerves in non-curarized muscles (Table II). Thus, it appears that at early times after partial denervation a moderate number of muscle fibers accept innervation from both nerves and that at later times most of this hyperinnervation disappears. There is not a clear trend for hyperinnervation as measured by muscle twitches (Table II). This could have a number of explanations. The intracellular recording measurements give estimates of hyperinnervation only in that portion of each muscle reinnervated by the soleus motor nerve. This portion on the average was only $\frac{1}{4}$ of the total tension in the 9 and 10 day survival muscles (Table II). Assuming a 20% hyperinnervation in this portion of the muscle, at 1/10 or so of the total muscle would be hyperinnervated. This small degree of hyperinnervation may well be beyond our limits of detection in tension occlusion measurement especially since we normally measure a small degree (up to 14%) of "apparent" convergence in normal adult muscles with aberrant and soleus nerves where in fact no convergence exists (Thompson and Jansen 1977). This inaccuracy in our measurement technique is apparent due to a lack of pure isometric conditions.

In addition to determining whether muscle fibers became innervated by both aberrant and soleus nerve fibers, we also determined by microelectrode recording and grading the stimulus to each nerve independently whether multiple axons in each made synapses onto the individual muscle fibers. Of 216 muscle fibers giving an epp to the aberrant nerve, none were polyneuronally innervated by the aberrant nerve. Hence, as previously reported (Thompson and Jansen 1977) the aberrant nerve fibers form single synaptic contacts upon sprouting. Of 330 muscle fibers giving an epp to the regenerating soleus nerve, only 14 fibers were polyneuronally innervated by the soleus nerve fibers. Even at the earliest times after soleus nerve regeneration only 4 of 93 fibers examined were found to be polyneuronally innervated. Hence, in contrast to the reinnervation of neonatal soleus muscle (Brown *et al.* 1976) and the reinnervation of an adult fast muscle of the rat (McArdle 1975) polyneuronal reinnervation of the soleus muscle is rare.

The sizes of the motor units in the muscles partially denervated by nerve crush were determined as described above and the results are presented in Fig. 5. The material has been divided according to the survival time following partial denervation. The sprouted motor units after nerve crush (Fig. 5 A) average size 315 fibers, did not become as large as those

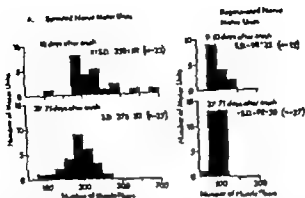


Fig. 5. Size of sprouted and regenerating motor units in muscles partially denervated by crush of the soleus nerve. The data are divided into two histograms according to survival time after nerve crush. Top histograms (left A & B) give the motor unit sizes for 4 muscles examined 9-10 days after nerve crush; the lower histograms (left A & B) give the motor unit sizes for 4 muscles examined 37-71 days after nerve crush. Motor unit sizes of the data presented here were compared using 3,400 fibers for the normal motor unit present in each muscle. Motor unit counts made of some of the muscles, as average of 3,340, as obtained.

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Further, the measurements of the sizes of the sprouted motor units indicate that these units may be decreasing in size with time after partial denervation. The sprouted motor units are larger at 9-10 days after partial denervation than they are at later times (37-71 days). This difference is statistically significant (Wilcoxon, $P < 0.01$), and suggests that some of the sprouting axons which have been fully functional lose in the competitive interactions with the regenerating axons. Presumably this applies to the muscle fibers which were doubly innervated at early times. This inference is further supported by the finding, shown in Fig. 5 B, that the motor units of the regenerating nerve do not significantly change in size.

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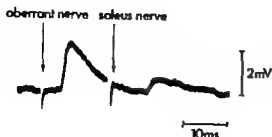


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The sizes of the motor units in the muscles partially denervated by nerve crush were determined as described above and the results are presented in Fig. 5. The material has been divided according to the survival time following partial denervation. The sprouted motor units after nerve crush (Fig. 5 A) average size 315 fibers, did not become as large as those in

parent exception to the rule that innervated and functionally active muscle fibers do not accept further innervation (Ekberg 1917). However it appears that the sprouting (Thompson and Jansen 1977) and regenerating (Gutmann and Young 1944) nerve fibers form their synapses at the old, denervated endplates on the rat soleus muscle fibers. These endplate sites have been shown to retain a special innervability even on muscle fibers which are fully active (Jansen *et al* 1973) or even innervated elsewhere (Frank *et al* 1975). The regenerating nerve fibers of the present material appeared to form their synapses adjacent to a sprouted nerve terminal within the old endplate site; thus, the hyperinnervation is in line with previous observations. Bennett and Ruiton (1977) have reported that sprouting nerves in axotomized muscles form synapses at denervated endplates which do not fully occupy the original site. These sites can then be reinnervated also by regenerating nerve fibers. This could be the case in the present experiments also.

The present results indicate that the amount of hyperinnervation is related to the time elapsed between partial denervation and reinnervation by the regenerating axons. If the regenerating axons began forming synapses in the muscle before the sprouting nerve fibers had consolidated their new end-plates, as after nerve crush, then a substantial number of the muscle fibers became hyperinnervated. If however regeneration occurred later after the sprouting nerve fibers had completed expansion, as after nerve cut, then very few muscle fibers became hyperinnervated. A simple explanation of this time dependence would be that the terminals of the sprouting nerve fibers require a certain period of occupancy of the endplate site before they can render the site refractory to further innervation, perhaps by growing completely over the synaptic area (Bennett and Ruiton 1977) or perhaps by rendering any empty synaptic space non-innervable (Frank *et al* 1975). There is, however, an inconsistency to this otherwise appealing view. Guth (1962) reported a much higher incidence of hyperinnervation in his partially denervated soleus muscles than what was found in the present material. In Guth's experiments the partial denervation was produced by spinal root section. His reinnervation would therefore be appreciably later than what we found after distal nerve section. Guth, however, assessed the degree of hyperinnervation solely from lesion measurements. As noted above there are potential complications with these recovery methods. Otherwise, I cannot explain the discrepancy between Guth and the present results.

The hyperinnervation was significantly reduced over a time course of less than one month. This means that one of the synaptic inputs on the hyperinnervated fibers was eliminated and suggests that the two nerve terminals interacted competitively for maintenance on each muscle fiber. The finding that the sprouted (aberrant) motor unit is significantly decreased in size during the time period over which the elimination occurred, while the regenerated motor units did not change in size, further suggests that it is the terminals of the sprouted nerve fibers which are losing out in the competition. It may well be that the expanded, large size of the sprouted motor units places their nerve terminals at a competitive disadvantage in relation to the regenerating axons, which support many fewer terminals in the muscle. A similar competitive advantage of small motor units over large motor units has been noted in the distribution of polynervous innervation from soleus muscle fibers of neonatal rats (Brown *et al* 1976). Brown and Irons (1977) have described cases of partial denervation of the mouse peroneus tertius muscle where a few remaining axons sprout to innervate all the

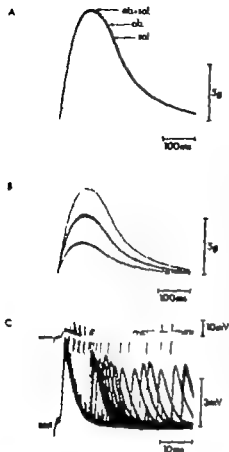


Fig. 6. Illustration of sprouting of aberrant nerve fibers in the soleus nerve distal stump. A segment of the soleus nerve had been resected 59 days earlier. Part A shows the twitch obtained to stimulation of the soleus nerve alone (sol), of aberrant nerve alone (ab), and both nerves together (ab+sol). The twitches to each nerve are almost indistinguishable and occlude completely. Part B also shows the 3 units just found in the soleus nerve close to the muscle; the aberrant nerve contained 3 identical units. Part C shows a recording of an endplate potential from a single fiber of the control (4 10^{-6} g/ml) muscle. Stimulation of the aberrant soleus nerves gave end-plate potentials which are easily distinguishable in rise time and amplitude. In part C the aberrant nerve is stimulated first and the soleus nerve at a variable latency later. If the soleus nerve stimulation occurred at 7 msec of the aberrant nerve stimulation, no response is obtained to the soleus nerve stimulation. Stimulation of the peripheral end of the original soleus nerve was effective due to the muscle while its central portion was inexcitable.

larger than when either nerve was stimulated alone. The number and sizes of the units in the soleus nerve were the same as those in the aberrant nerve (Fig. 6 B). Finally microelectrode recordings from the muscle fibers revealed that each fiber in which an action potential (or epp) was evoked by stimulating one nerve also had an action potential (or epp) evoked by the other nerve. Further, as shown in Fig. 6 C, the epps occluded each other if the second stimulus followed the first by less than about 7 ms. This is in contradistinction to the hyperinnervated muscle fibers shown above, where epps to the two nerves summated and could not be occluded. Therefore, in these experiments the cut nerve had apparently not regenerated to the muscle. Rather sprouts of the aberrant nerve fibers had entered the peripheral stump of the soleus nerve and were growing back in a proximal direction. It is well-known that sheaths, tubes, and perhaps products of degeneration of isolated nerve segments form an excellent attractant and conduit for nerve growth (Ramon y Cajal 1959). This appears to be the explanation for the present finding.

Discussion

The results confirm the observations of Guth (1962) that nerve fibers regenerating to a partially denervated rat soleus muscle can hyperinnervate muscle fibers which are already functionally innervated by sprouted nerve fibers. These results, therefore, constitute a

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denervated fibers. Nevertheless, upon regeneration of the interrupted axons, some of the muscle fibers initially become dually innervated. Subsequently the dual innervation is lost by elimination of the sprouted nerve terminals and there is a reappearance of muscle fibers innervated exclusively by the regenerating axons. What may be similar cases of competitive disadvantages of sprouted nerve terminals in relation to regenerating nerve terminals have been reported by Betz (1977) in a rat foot muscle, by Roper (1976) in a parasympathetic ganglion of the frog, by Cass *et al* (1973) and Bennett and Raptos (1977) in axolotl trunk and by Guth and Bernstein (1961) in a mammalian sympathetic ganglion.

The elimination of hyperinnervation which occurs in these muscles may be contrasted with the lack of a similar elimination observed by Frank *et al* (1975). Frank *et al* transplanted foreign nerves to the proximal end of the soleus muscle. These foreign implants established synapses at ectopic sites on the muscle fibers when the original innervation of the muscle was interrupted. If the interrupted axons regenerated quickly they were able to reinnervate almost all of the muscle fibers by establishing synapses at the old endplates. As a result, most muscle fibers were hyperinnervated by the foreign nerve at an ectopic site and by the original nerve at the old endplate. This hyperinnervation persisted indefinitely. Two possible reasons can be offered for the elimination of hyperinnervation seen in the present study. First, it could be that the interactions necessary for synapse elimination can occur only for a limited distance along each muscle fiber as suggested by the experiments of Kuffler *et al* (1977). In the present experiments the new synapses were all formed at or very close to an original endplate. In the experiments of Frank *et al* on the other hand, the synapses were located several mm apart, perhaps too far for the competitive interaction to act. A second, alternative explanation of the difference would be that the competition in the present experiments was for occupancy of a defined synaptic area (the old endplate site), while in the experiments of Frank *et al* there was no such competition.

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third ventricle (*cf.* Anderson 1977). Therefore, it has been of interest to cover this area with a field of bipolar electrical stimulation in the conscious goat. The use of thermo-couple electrodes has made it possible to study also water intake during moderate, controlled heating within the medial forebrain. This has been of interest primarily because more intense heating of this part of the brain earlier was found to cause drinking in the goat (Anderson and Larsson 1961).

Methods

Animals. Local anesthesia was made in 13 female goats (b. wt. 35 kg). Four of these animals were used for electrical stimulation. The goats were kept in metabolism cages at room temperature ($20 \pm 1^\circ\text{C}$). Here all the electrical stimulations and most of the heating experiments were conducted. The animals received hay *ad lib* and had their water buckets refilled *ad lib* every day. The experiments were started about 10 months when the goats had commenced hay and fresh water to satisfaction.

Electrode implantations. All goats had pairs of stainless steel thermo-couple electrodes (ϕ 0.7 mm) craniotomically implanted through the skin into the preoptic anterior hypothalamic region for the final purpose of making radio-frequency (RF) lesions. The implantations were performed under general (nembutal) anesthesia about 2 weeks before any experiments were started. The two electrodes were either placed along the midline of the brain with an interspace of 3 to 5 mm, or bilaterally with an interspace of 3 or 4 mm. The length of the insulated tips of the electrodes varied between animals from 3 to 5 mm.

Electrical stimulations. A modified Hov's technique, earlier described in detail (Anderson, Pernow and Olsson 1969) as employed bipolar as well as unipolar brain stimulations were performed. In the latter case subcutaneous steel wires on the animal's neck served as the reference electrode. The following stimulus parameters were used. Current strength 0.4–4 mA, pulse frequency 50/s, and pulse duration 1.5 ms. The stimulation periods were 1 to 10 min.

Radio-frequency (RF) warming. A steady 2°C elevation of the temperature of the brain tissue between the electrodes was achieved by applying RF-energy between the insulated ends of the thermo-couple electrodes, leaving their temperature recording units connected to an "Elsh" thermometer. The duration of the warming periods was 40 min. For further details about the RF-technique employed see Gale (1963).

Bloods. Occasionally electrical and thermal stimulations were performed in the pre-hydrated goat about 90 min after the administration of 60 or 100 ml/kg of 32°C water into the rumen by stomach tube.

Urine sampling. During experiments in hydrated animals urine was collected in fraction collector for determination of flow and osmolality (by use of an Advanced Instruments Inc. osmometer).

Histology. At the termination of the experimental series involving electrical and thermal stimulation, the electrodes were used for producing RF-lesions in the medial forebrain of all goats. At the end of the post-lesion observation periods the animals were decapitated under nembutal anesthesia and the heads were perfused with physiological saline followed by 8 formal saline. After formal fixation, block of the brain (including the preoptic region, the thalamus and the hypothalamus) was embedded in celloidone and cut by serial transverse sections at 50 μm . The sections were stained either with toluidine blue or according to Loewen (*cf.* Callahan 1957).

Results

Electrical stimulations

Of the 4 animals subjected to electrical stimulation, 2 goats (here named A and B) had bilaterally implanted electrodes (3 mm apart, uninsulated ends 3 mm). On preliminary bipolar stimulation both animals characteristically responded with post-stimulatory water consumption, and their diuretic responses were subjected to continued analysis over periods of 5 and 3 weeks respectively. The other two animals had their electrodes placed along the midline of the brain. Here bipolar electrical stimulation did not induce drinking. However in one of these goats (goat C) strong (4 mA) unipolar stimulation via the anterior electrode (Fig. 3) elicited drinking after a latency time of about 30 s. Since

Delayed drinking in response to electrical and thermal stimulation of the medial forebrain

By

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Abstract

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Water intake in response to electrical and thermal stimulation of the medial forebrain was studied in the goat. When the frontal wall of the third cerebral ventricle was included in a field of bipolar electrostimulation a dipsogenic response was obtained after discontinuation of the stimulation. Release of antidiuretic hormone (ADH) was apparently also elicited. The water intake was roughly proportional to the amount of current which had been applied during the stimulation period. Water consumption in response to stimulation attenuated the dipsogenic effect of subsequent stimulation as did also pre-stimulation hydration by stomach tube. A 2°C elevation of the temperature of parts of the preoptic/anterior hypothalamic region for 40 min periods induced cumulative drinking starting after 5 to 18 min. There were great interindividual differences in the amount of water consumed in response to the thermal stimulation, possibly due to variations in thermo-couple electrode placement. The dipsogenic effect of forebrain warming was inhibited by pre-hydration, and by lowering of the environmental temperature. The delayed drinking responses are discussed in relation to stimulus-bound drinking previously observed in the same and other species. It appears possible that the delayed drinking was a manifestation of artificially induced excitation of juxtaventricular "thirst" receptors.

Verney's (1947) fundamental studies in conscious dogs conclusively demonstrated that the cerebral sensory mechanism plays an important role in the control of water balance by regulating the release of antidiuretic hormone (ADH) from the neurohypophysis. His investigations also implied that the sensitive brain cells primarily are excited by a reduction of their own volume and are located in the anterior hypothalamus. This osmoreceptor concept was extended to involve also the regulation of water intake when it was found that injections of hypertonic NaCl into the anterior hypothalamus elicited excessive drinking in water-replete goats (Andersson 1953). Electrical stimulation was later employed to study the cerebral thirst mechanism, and it was found that strictly stimulus-bound drinking could be elicited by unipolar or bipolar stimulation within the perifornical region in the goat (Andersson and McCann 1955), and the lateral hypothalamus of the rat (Greer 1955). However, the integrated results of later studies imply that hypothalamic receptors regulating water intake and ADH-release are located more medially close to the anterior border of

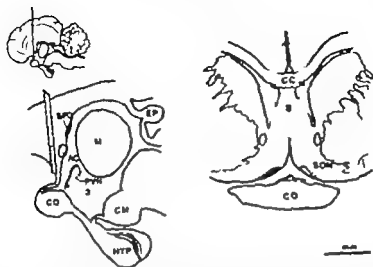


Fig. 2. Position of the bilaterally implanted electrodes in goat A shown on midsagittal (left) and coronal (right) sections. The 3 mm uninsulated ends of the electrodes are hatched. AC: anterior commissure, CC: corpus callosum, CM: corpus mammillare, CO: choroid opticus, EP: epiphysis, HYP: hypothalamus, MI: massa intermedia, PVM: periventricular nucleus, S: septal region, SFO: subfornical organ, SON: supraoptic nucleus, 3: third ventricle.

strength was then generally limited to 0.5 mA. When goat B was not pre-hydrated (6 sessions) the initial trials (0.5 mA for 5 or 10 min) invariably induced drinking (200 to 400 ml) about one min after the stimulation had been switched off. Repeated stimulations of the same strength and duration did not induce further water consumption. Likewise, pre-hydration (2 sessions) prevented post-stimulatory drinking.

Apparent release of antidiuretic hormone (ADH). The bipolar stimulations which caused delayed drinking apparently also resulted in release of ADH as indicated by the results shown in Fig. 1 (right). Here goat A had developed positive renal free water clearance" as a consequence of over-drinking after preceding periods of stimulation (Fig. 1 left). Repeated stimulations then caused temporary inhibition of the water diuresis and the degree of inhibition was proportional to stimulus duration. Similar graded, antidiuretic effects were obtained when the animal had been hydrated by stomach tube before the experimental session started.

The threshold stimulus (0.5 mA) for delayed drinking in goat B was ineffective, not only in eliciting drinking, but also in inhibiting the water diuresis when this animal was pre-hydrated. However an antidiuretic effect could then be obtained in response to more intense stimulation (1 mA for 5 min).

Electrode location. The position of the electrodes in goat A is shown in Fig. 2. It is evident that electrical impulses passed between the uninsulated ends of the electrodes must have affected brain tissue close to the anterior border of the third ventricle. This is also indicated by the fact that the RF-lesion later induced in this goat included about two thirds of the anterior wall of the ventricle and extended 2 mm in front of the anterior

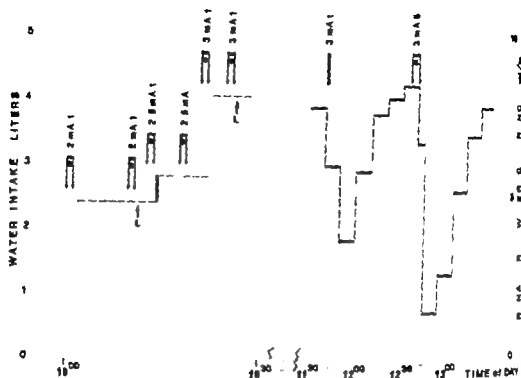


Fig. 1 *Left* Cumulative post-stimulatory drinking in response to repeated electrical stimulations in goat A. Note the rise in stimulus threshold for drinking after previous water consumption. *Right*: Stimulations causing temporary inhibition of the water diuresis which had developed in consequence of the previous cumulative drinking. No drinking occurred in response to the relatively strong stimulation, but the animal was hydrated to this extent. No stimulations were performed during the interval (10:30–11:30). Current strength and duration above each stimulation period (S). L = Licking in the water.

stimulation at this strength also induced restlessness further studies of the effects of electrical stimulation were omitted.

Drinking after bipolar stimulation Goat A was subjected to consecutive electrical stimulations on 17 daily sessions over a period of 5 weeks. When not pre-hydrated (15 days) the lowest intensity of stimulation needed to induce the initial thirst response was either 1 mA for 5 min, or 2 mA for 1 min. Preceding stimulations of lower strength and/or shorter duration (e.g. 0.5 mA for 10 min, or 1 mA for 1 min) were found ineffective. Drinking generally commenced precisely half a min after the discontinuation of stimulation and 1430 ± 210 ml of water was then drunk in one sequence. On repeated trials in the next session stronger stimuli were needed to induce further drinking as demonstrated in Fig. 1. Here, and in other sessions, the goat did no longer drink after intense (3 mA, 1 and 5 min) stimulation when preceding stimulations had induced a total water intake of about 4 l. Similarly during the two sessions when goat A was pre-hydrated (water load 60 ml/kg) the stimulus threshold for drinking was elevated. The duration of post-stimulatory thirst was studied on two occasions by temporarily preventing the animal from drinking. The experiments revealed that the urge to drink was still present 9 min after stimulation.

In goat B bipolar stimulation at 1 mA, or stronger induced restlessness and shivering. For that reason the animal was only subjected to 8 experimental sessions and the stimulus

was performed when the goat was either pre-hydrated ($n=2$), or transferred to a cold (4°C) environment 1 h prior to the start of central warming ($n=2$). Under these circumstances no water was consumed. Furthermore, when the animal was pre-hydrated (10 ml/kg) the forebrain warming obviously did not cause significant release of ADH, nor any reduction of the water diuresis occurred.

Electrode location. In goat C the electrodes were placed along the midline of the brain. The position of their 5 mm unstimulated ends is shown in Fig. 3. It appears that most of the anterior wall of the third ventricle had been subjected to warming during the application

of RF-energy sufficient to cause a 2°C elevation of the temperature at the tips of the electrodes. An idea about the approximate extent of the warming was provided by later F-lesioning (raise of temperature to 60°C for 4 min) which resulted in the destruction of almost the entire anterior wall of the ventricle including most of the subfornical organ and the supraoptic crest. The bilateral extent of this lesion, which made the animal adipsic, is shown to the right in Fig. 3.

Discussion

Drinking as effect of electrical stimulation at various sites in the hypothalamic region has been reported in several mammalian species over the past 22 years (cf Fitzsimmons 1977). Generally the effective sites of stimulation have been at the sagittal level of the descending column of the fornix, or further laterally and the dipsogenic effect has been rather strictly stimulus-bound. In the goat (Anderson and McCann 1955) drinking as effect of perifornical stimulation was not attenuated by water consumption. Therefore, conspicuous over-hydration could be induced by frequently repeated, or prolonged periods of stimulation, and it was assumed that this polydipsia was the manifestation of hyperactivity in a hypothalamic "thirst center". However the results of recent studies imply that cerebral receptors regulating water intake predominantly are located more medially in, or close to the anterior wall of the third ventricle (cf Anderson 1977). For this reason it appears unlikely today that the stimulus-bound drinking previously obtained at more lateral sites in the hypothalamic region was due to excitation of cerebral "thirst receptors". A more probable explanation seems to be stimulation of pathways transmitting impulses from receptor areas to parts of the brain where the urge to drink is integrated. Some support for this view is provided by the results of the present study.

Stimulus-bound drinking was not elicited when the presumptive cerebral "thirst receptor" area was subjected to field-stimulation. Rather unexpectedly however thirst became apparent half to one min after cessation of stimulation. When not satisfied, the urge to drink persisted for several minutes. Furthermore, like normal thirst, it was inhibited by dilation of the body fluids (pre-hydration). Apparently temporary field-stimulation of brain tissue bordering the third ventricle in some manner induced post-stimulatory activity similar to that developing in this area during dehydration. However it is impossible to tell whether this activity was a manifestation of events at the receptor level, or consisted of post-stimulatory epileptic discharge in neurons normally processing receptor information. Of interest with regard to the latter possibility are reported effects of electrical stimulation within the dorsal vagal nucleus in the goat (Anderson, Kitchell and Pearson 1958). Normal

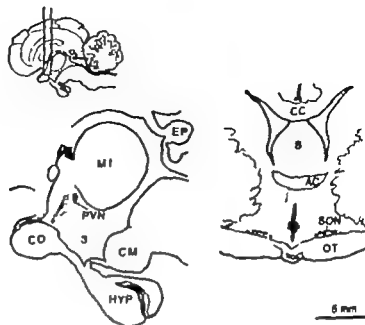


Fig. 3 *Left*: Midsagittal section showing the location of the electrodes in goat C (implanted along the midline of the brain). This animal consistently responded with drinking to RF-warming (2°C) between 5 mm uninsulated ends of the electrodes (hatched). *Right*: Transverse section at the level of the third commissure showing the bilateral extent (hatched area) of the RF-lesion later produced in this animal. Abbreviations. See Fig. 2. Additional. OT=optic tract.

commissure. The position of the electrodes in goat B has not yet been verified by histological examination. However judging from X ray pictures taken during the implantation procedure, the uninsulated ends of the electrodes had a location very similar to what is shown in Fig. 2. An indication that the stimulations in goat B involved a cerebral thirst receptor area was provided by the fact that the RF lesioning induced permanent adipsia.

Forebrain warming

Water Intake Prior to RF-lesioning all animals were subjected to a preliminary experiment in which the effects of local forebrain warming (1°C for 40 min) were studied. As expected from the studies by Magoun *et al* (1938) and later investigations of cerebral control of body temperature, all animals reacted with peripheral vasodilatation and polydipsia to a varying degree. 5 of the goats also drank during the first warming period. Since drinking occurred also in additional experiments it appears safe to conclude that it was a true effect of the forebrain warming. However the latency time for thirst varied between animals from 2 to 18 min and there were also great interindividual variations in the amounts of water consumed. Furthermore the water intake was apparently not proportional to the intensity of polydipsia.

One of the responding animals (goat C) was selected for a further analysis of the dipsogenic effect. When maintained at room temperature, and not pre-hydrated (expt. I) goat C invariably responded with drinking during the 40 min forebrain warming. The average latency time for drinking was 6.7 ± 1.6 min and the mean cumulative water intake was 1280 ± 650 ml. In between these positive experiments, identical forebrain warming

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reticulo-ruminal motility was completely arrested during the stimulation period, but conspicuous, and rather persistent hyperactivity developed almost immediately after.

Available information on the nature of juxtaventricular receptors involved in the control of water balance (cf Andersson 1977) appears of interest considering eventual influence at the receptor level of the present electrical and thermal (see below) stimulations. Cerebral receptors regulating water intake and ADH-release are obviously sodium-sensitive and some recent studies vaguely indicate that Na-transporting enzyme activity forms a link in the excitation process (Olsson, Larsson and Liljekvist 1976, Rundgren *et al* 1977). The possibility exists that the present field-stimulation induced transmembranal redistribution of Na and other cations. Local redistribution of Na could have acted like a physiological stimulus for thirst and ADH release when the pulse train no longer interfered with the normal function of the brain area in question. This would explain why post-stimulus thirst was susceptible to the normal inhibitory influence of hydration.

Heating of the preoptic/anterior hypothalamic region via large medially implanted thermodes was earlier found to induce drinking in the goat concomitant with mobilization of heat dissipation mechanisms (Andersson and Larsson 1961). The validity of this observation as evidence for a suggested physiological interaction between cerebral mechanisms regulating body temperature and water intake is obscured by the fact that the thermode temperature was elevated by 5°C or more. Heating to that extent might have acted as a noxious stimulus of structures of importance for the development of thirst. In the present study drinking was elicited by a moderate (2°C) elevation of the medial forebrain temperature, which hardly could have been a noxious stimulus. This, however, provides no additional evidence for an interaction of the kind mentioned above, since there was no correlation between the amounts of water consumed and the degree of polydipsia. Other events (e.g. blood pressure elevation) may well explain the fact that the dipsogenic effect vanished during acute exposure to external cold.

Why moderate elevation of the temperature in the medial forebrain elicited drinking remains an open question. At present, any attempt to answer it must be highly speculative. However, the presumptive cerebral "thirst receptor" area was subjected to warming in the positive experiments (Fig. 3). Due to the van t Hoff effect, the elevated temperature could have induced increased enzymatic activity in that area. Provided Na-transporting enzyme activity really is essential for excitation of cerebral "thirst receptors" (see above), the metabolic effect of warming could explain the animals' urge to drink.

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2.1. Description of rabbit diets

Sodium chloride content (%)	Source
0.25	Altromin GmbH Lager, Uppre, West Germany
2.5	Altromin GmbH Lager, Uppre, West Germany
0.57	Astra-E on AB, Södertälje, Sweden
11	Astra-E on AB, Södertälje, Sweden
0.43	Astra-E on AB, Södertälje, Sweden

lary PGs originate within the kidney (Frölich *et al.* 1975, Williams *et al.* 1977) and their measurement therefore constitutes an index of intrarenal biosynthetic activity. Our results show that sodium deprivation markedly enhances renal excretion of PGs pointing to the possibility of an antinatriuretic role of the PGs.

Material and Methods

rabbits are nonpregnant albino weighing 2.5 kg at the start of the study. They are quarantined on one of the diets shown in Table 1.

The animals are kept in plastic cages with one rabbit in each cage. The 4-hour urines from each cage were collected separately. Special care was taken to avoid degradation of the PGs by collecting urine in traps surrounded by dry ice. The urine was then frozen very soon after being voided and kept until analysed.

Experimental protocol. Two experimental designs were used. In the first the rabbits were fed the normal (1), high sodium diet (2) and low sodium diet (3) *ad libitum*. On each diet animals were allowed to stabilize for one week and then urine was collected for 4 days. It was observed that intake of both food and water varied considerably between the high sodium diet and the other dietary treatments. With the low sodium diet the food intake was 3-4 times that of the high sodium diet. A second series of experiments was therefore decided to give the animals different amounts of sodium while keeping the intake of food constant.

The diets were given in the following order: high sodium diet (4) for 6 days, normal diet (3) for 6 days and low sodium diet (1) for 6 days. The first 3 days were allowed for equilibration and urine was collected only for another 3 days.

Analyses. Urinary sodium and potassium was measured using flame photometry (Perkin-Elmer Laboratory Inc. Model 143).

PGF_{2α} in urine was measured by radioimmunoassay (RIA). This procedure has been described in detail elsewhere (Olwe *et al.* 1978b). Briefly, the precision of the method was evaluated by replicate (n = 4) measurement of the same samples. The coefficient of variation was determined on 4 separate occasions and found to be 10.7%, 13.4%, 12.7% and 18.5% (mean 13.8%). The validity of the method was assessed by co-determination of 6 different samples with gas chromatography-mass spectrometry (GC-MS) using synthetic PGF_{2α} as an external standard. The results are shown in Table II. It is seen that the values obtained by the two methods are in close agreement, thus demonstrating the validity of the RIA method.

In experimental design 1 PGE₂ was released by NaBH₄ to PGF_{2α} and PGF_{2α} and PGF_{2α} measured by RIA using PGF_{2α}-antiserum (Olwe *et al.* 1978b). In experimental design 2 PGE₂ was determined by incubation on the rat ileum strip according to Larsson and Ånggård (1974). To 20 ml of whole urine was added 50 000 cpm of H-labelled PGE₂ (100-200 Ci/mole, New England Nuclear, Dartmouth, West Germany). After adjusting the pH to 3 using 20-40 drops of 30% formic acid, the sample was extracted in ice with 20 ml of CHCl₃. The solvent was evaporated to dryness and applied on a 1 g

The influence of dietary sodium on urinary prostaglandin excretion

By

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Abstract

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The influence of dietary sodium chloride on the urinary excretion of prostaglandins (PGs) was studied in unanesthetized female rabbits housed in metabolic cages. Urinary PG levels were determined by radioimmunoassay, bioassay and gas chromatography-mass spectrometry. In the first experiment rabbits were fed at high (2.5%) and later a low (0.25%) sodium chloride diet *ad libitum*. A 2-5 fold increase in excretion of immunoreactive $\text{PGF}_{2\alpha}$ (IPGF $_{2\alpha}$) and IPGE_2 was noticed when animals were given the low salt diet. Since it could not be excluded that dietary factors other than sodium chloride contributed to the change, a second more controlled experiment was undertaken. Rabbits were fed 30 g/kg per day of diets differing only in the content of sodium chloride, 2% and 0.37% respectively. On the high salt diet the rabbits excreted $0.1 \pm 0.04 \mu\text{g/day}$ of PGE_2 and $0.0 \pm 0.5 \mu\text{g/day}$ of IPGF $_{2\alpha}$. After equilibration on the low salt diet the PGE_2 excretion rate increased to $1.5 \pm 0.3 \mu\text{g/day}$ ($p < 0.001$) and that of IPGF $_{2\alpha}$ to $3.4 \pm 0.4 \mu\text{g/day}$ ($p < 0.001$). These results thus point to an inverse relationship between renal sodium excretion and the activity of the renal prostaglandin system.

The role of the prostaglandins (PGs) in the renal handling of sodium is unsettled. Earlier studies using infusion of E and A types of PG demonstrated natriuretic and diuretic effects (Herzog *et al.* 1967, Vander 1968, Leo 1973). Similarly an infusion of arachidonic acid, a PG precursor into the renal artery caused natriuresis in doses which did not alter renal blood flow or glomerular filtration rate (Chang *et al.* 1975, Tannenbaum *et al.* 1976, Bolger *et al.* 1976). On the other hand meclofenamate increases renal sodium excretion both in the conscious and in the anesthetized dog in response to volume expansion (Kirschenbaum and Stein 1976, 1977, cf. Olaw *et al.* 1978a). Furthermore, Tobian *et al.* (1974) and Tobian and O'Donnell (1976) have shown that sodium loading in rats depresses intrarenal concentration of PGE_2 , whereas deprivation has the opposite effect. Quite recently Webb *et al.* (1977) reported that a high sodium diet depresses the renal excretion of PGE_2 in the rabbit.

In the present study we have attempted to evaluate the effect of the dietary sodium on the renal excretion of PGs in the conscious rabbit. As has been shown earlier the present

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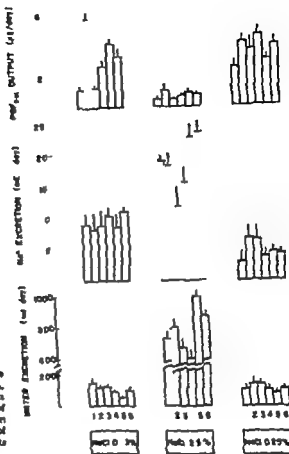


Fig. 1 The effect of dietary sodium on the urinary excretion of immunoreactive PG F_{1-4} , sodium and water in 6 different rabbits *ad libitum*. The representations (means of days \pm S.E.) of variables on each diet were made after an equilibration period of 48 h. Figures under the bars denote each individual rabbit.

could possibly alter the renal output of PGs by mechanisms unrelated to renal handling of sodium. In the second series of expts. the rabbits were restricted to a food intake of 30 g/kg/day. Water was offered *ad libitum*. The animals were first given the high sodium diet (4) and then low sodium diet (3) and measurements were made after three days of equilibration on each diet. The results are shown in Fig. 2. Again an inverse relationship was found between PG excretion and sodium output. The values for urinary volume, pH and output of sodium were constant between the two dietary regimens. The changes in IPGF_{1-4} and PGE_2 excretion were significant ($p < 0.01$ and $p < 0.001$ respectively).

Discussion

The most apparent finding of the present study was that the urinary output of PGs was dependent on dietary sodium. An involvement of renal PGs with the renal handling of sodium was previously suggested by Tobian *et al* (1974) and by Tobian and O'Donnell (1976). These authors noted an elevation of the intrarenal levels of PGE_2 but not of PGF_{1-4} .

TABLE II Comparison of PGE_{2x} determination by radioimmunoassay and gas chromatography-mass spectrometry in urine.

Sample	RIA ng/ml	GC MS ng/ml	Difference
1	36	36	0
	21	34	38
3	37	37	0
4	20	23	13
5	27	28	4
6	3	39	18
Mean \pm S.D.	29 \pm 7	33 \pm 6	12 \pm 14

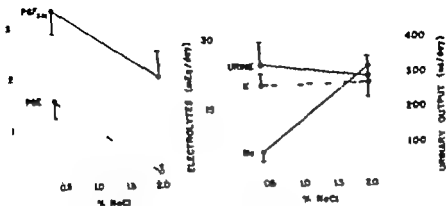
silicic acid (Unisil, 100–200 mesh, Clarkson Chem. Comp. Inc., Williamsport, Pa., USA) column in ethyl acetate in toluene. After 30 ml has been allowed to pass through the column the PGE_{2x} was eluted using 60 ml of 60% ethyl acetate in toluene. This fraction was evaporated to dryness, dried in 1.5 ml of 96% ethanol and kept at -20°C until assayed. The recovery of ^3H PGE_2 through this elution procedure was about 30% (range 25–57%). The recovery of added 50 ng of PGE_2 was assessed before and after correction of losses of 11 PGE_2 found to be 98%. Prior to bioassay the samples were reduced to dryness and reconstituted in 1 ml of Tyrode solution. The assay of the rat headless strip is carried out according to Weeks *et al.* (1968). The validity of the assay was further checked by treatment of the strip with dilute NaOH. This procedure, which converts all PGE-compounds to the respective PGB-compound, resulted in complete biological inactivation. The level of the PGE_2 present in the original sample obtained using the data from the bioassay and the recovery of radioactive PGE_2 in each sample. Statistical significance was determined by Student's *t*-test.

Results

In the first experiment rabbits were fed a normal diet, a high (2.5%) and a low (0.2%) sodium chloride containing diet *ad libitum*. The excretion of prostaglandins, sodium and water is shown in Fig. 1. An inverse relationship was found to exist between the excretion of sodium on the one hand and of IPGF_{2x} on the other hand.

When the rabbits were fed the normal rabbit chow diet (5) containing 0.43% NaCl a 10-fold interindividual variation in output of IPGF_{2x} was seen. However the intra-individual variation was relatively small (cf. Fig. 1). The mean excretion of IPGF_{2x} was $2.9 \pm 1.7 \mu\text{g/h}$ (mean \pm S.D.). Change to the higher sodium diet led to a decrease in the renal output of IPGF_{2x} to a mean value of $0.69 \pm 0.3 \mu\text{g/24 h}$ but increased both sodium and water excretion. When during the last stage of the expts. the rabbits were eating the diet with the lowest content of sodium the urinary PGF_{2x} excretion again increased to a mean level of $3.5 \pm 0.7 \mu\text{g/24 h}$. The mean level of IPGF_{2x} and IPGE_2 for the two later diets are shown in Table III. The differences on PG excretion between the two diets were statistically significant (both $p < 0.001$).

It thus appeared that there might be an inverse relationship between the renal excretion of sodium and the excretion of IPGF_{2x} and IPGE_2 . However two factors led us to undertake a second more controlled experiment. Firstly the rabbits ate 3–4 times more of the low sodium diet as compared to the high sodium diet. Secondly the rabbits drank roughly 3 times more during the high salt diet as compared to the low salt diet. These differences



2. Effect of dietary sodium (2 and 10 mmol/kg day) on the excretion of PGE₂ measured by two-way (on same subjects) PGE₂ (top), sodium, potassium and water (bottom) (restricted 30 g/kg day but not star as offered ad libitum). T and three asterisks denote difference between two diets at $p < 0.01$ and $p < 0.001$ respectively. Means \pm S.D.

74), with anesthetized dogs (McGill *et al.* 1970 Williams *et al.* 1977) and conscious humans (Rohlf *et al.* 1975) have demonstrated increased release of PGs following infusion of angiotensin II. Moreover direct application of angiotensin II to slices of rat renal papilla *in vitro* causes release of PGs into the incubation medium (Danon *et al.* 1975 a). A second possibility could be that the renal medullary arachidonic acid metabolism is sensitive to variations in ADH or in medullary osmolality as indicated by the work of Danon *et al.* (1975 b), who reported elevated PG output from slices of rat renal papilla in response to hypertonic media. The third and perhaps most interesting alternative would be that the increased levels of urinary PGs are consequence of their involvement in a tubular sodium conserving mechanism as has been suggested by Tobian and O'Donnel (1976). Both the increased intratubular levels of PGE₂ during low salt intake observed by these authors, and the increased urinary excretion of PGs observed by us and the similar results by Weber *et al.* (1977), could be in accordance with this hypothesis, which, however must be regarded as tentative and awaits exploration in further experiments.

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TABLE III Excretion of immunoreactive $\text{PGF}_{2\alpha}$ ($\text{IPGF}_{2\alpha}$) and PGE_2 in response to variation in salt. Food and water offered *ad libitum*.

Diet	Mean \pm S.D. $\text{IPGF}_{2\alpha}$ ($\mu\text{g}/24 \text{ hrs}$)	Mean \pm S.D. PGE_2 ($\mu\text{g}/24 \text{ hrs}$)
(2.5 NaCl)	0.69 ± 0.3	0.46 ± 0.1
1 (0.06 NaCl)	3.50 ± 0.7	1.20 ± 0.3

$p < 0.001$

when rats were subjected to a low salt diet (0.06%) and a depression of PGE_2 on a high salt diet (10%). Their data are however open to alternative interpretations since it could be argued that the low levels seen during the high salt diet could be due to enhanced utilization of the endogenous precursors *in vivo* with a resulting low level of post mortem accumulation of PGE_2 in the kidney. Conversely the high level of PGE_2 seen post mortem is so on a low salt diet could be due to low activity of the PG system *in vivo*.

Our data collected in a different species and in a manner circumventing the difficulties discussed above nevertheless confirm and extend the observations of Tobian and O'Donnell (1976) that a decreased sodium intake is associated with an increased activity of the renal PG system or with a shift in arachidonic acid metabolism.

Weber *et al* (1977) have in a study independent of ours reported a similar inverse relationship between dietary sodium and urinary output of PGs in the rabbit with one important difference. These authors found a significant change only in the output of PGE_2 , but not in $\text{PGF}_{2\alpha}$. They showed that the intrarenal levels of the PGE_2 -9-ketoreductase was stimulated by the high salt intake and the authors suggested that the increased formation of PG could play a role in regulating renin release. In the present study clearcut and statistically highly significant changes were seen both in $\text{PGF}_{2\alpha}$ and PGE_2 excretion rates. The increase in $\text{PGF}_{2\alpha}$ excretion was seen both when the analyses were performed with radioimmunoassay and with gas chromatography-mass spectrometry and can not be due to limitations of methodology. The reason for this discrepancy can not be explained at present.

In the first experiment, similar in design to that of Weber *et al* (1977), the animals were fed *ad libitum*. It was noticed that these animals ate and drank differently on the high and low salt diets. It could be argued that the changes in urinary PG excretion could be due to different intake of other food constituents than sodium or due to differences in water excretion. The second experiment was better controlled since the intake of food was standardized (30 g/kg/day) and no significant changes were seen in water excretion. The results from the experiment therefore provide further proof of an inverse relationship between urinary PG and sodium excretion. A third possibility for changes in renal output of PGs, which are unrelated to sodium excretion could be changes in urinary pH. PGs are weak acids and their excretion could conceivably be influenced by nonionic diffusion. This possibility could however be excluded since similar urinary pH values were observed during both diets.

There are several alternatives to explain our results. One is that the increased circulating angiotensin II levels after renin release in the sodium restricted animals could stimulate intrarenal formation of PGs. Other studies with perfused rabbit kidney (Gagnon *et al*

Nicotine inhibits the release of 6 keto-prostaglandin $F_{1\alpha}$ from the isolated perfused rabbit heart

by

ÅKE WEN MÅLM

Prostaglandin I (prostaglycin, PGI_2) is a newly discovered prostaglandin with potent vaso-dilator and platelet anti-aggregatory properties (Gryglewski *et al* 1976, Johnson *et al* 1976). Prostaglycin is formed in vascular tissues from various organs and species (Gryglewski *et al* 1976). The compound degrades spontaneously in aqueous solution to its stable meta-olite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto- $PGF_{1\alpha}$) (Johnson *et al* 1976). This was recently shown to be the major prostaglandin released from the perfused rabbit and rat heart (Oakman *et al* 1977, De Deckere, Nigam and Ten Hoor 1977), and it has been suggested that the compound protects the coronary circulation against the formation of blood platelet aggregates (De Deckere *et al* 1977). Earlier studies in our laboratory demonstrated that the synthesis of PGE in the isolated rabbit heart is stimulated by nicotine (Wenmalm and Jonstad 1976, Wenmalm 1977). We now report that nicotine inhibits the formation of PGI_2 in the rabbit heart, as reflected by the appearance of 6-keto- $PGF_{1\alpha}$ in the cardiac effluent.

10 isolated rabbit hearts were perfused according to Langendorff with heated and gassed Tyrode solution. 3H -arachidonate (New England Nuclear 3 μCi , sp act. 60 Ci/mole) was infused at a rate of 0.25 $\mu Ci/min$ during two 10 min periods separated by a 10 min interval. The cardiac effluent was collected during the infusion of isotope and for the following 5 min. Nicotine was added to the Tyrode solution after the end of the first effluent collection period to produce final concentrations of $3 \cdot 10^{-5}$ – 10^{-4} M. Perfusion with nicotine containing solution was maintained during the second isotope infusion and to the end of the collection of effluent. The effluents from the hearts were extracted twice at pH 3–3.5 with an equal amount of ethyl acetate. After evaporation, the residue was subjected to thin layer chromatography (TLC) using 0.25 mm DC Fertigplatten Kiesel F 254 (Merck) in solvent ethyl acetate-acetic acid-2,2,4-trimethylpentane-water (90:20:30:100) (Humbert and Samuelsson 1966), against standards of 6-keto- $PGF_{1\alpha}$, $PGF_{1\alpha}$, PGE_2 , PGD_2 , PGA and Na-arachidonate. Radio scans of the chromatograms were developed, using a Berthold Dinnachuchi-Scanner.

During the infusion of isotope $75 \pm 3\%$ (mean \pm S.E., $n = 10$) of the radioactivity was retained in the organ. A major portion of the activity in the effluent ($55 \pm 6\%$) was extracted with ethyl acetate. The radioactivity released during the perfusion with nicotine-free Tyrode

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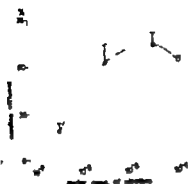


Fig. 2. Inhibitory effect of different concentrations of nicotine on the outflow of 6-keto-PGF_{1α} in the cardiac effluent. Ordinate: percent inhibition of the ratio between the amplitude of the 6-keto-PGF_{1α} peak and the mean amplitude of the PGE₂ and PGF_{2α} peaks, compared to controls. The control ratio, corresponding to 100 in the figure, is 0.91 ± 0.06 ($n=10$). This was obtained in effluents from hearts not treated with nicotine.

acid anti-aggregatory PGI₂ may constitute a biochemical basis for the link between tobacco smoking and cardiovascular disease.

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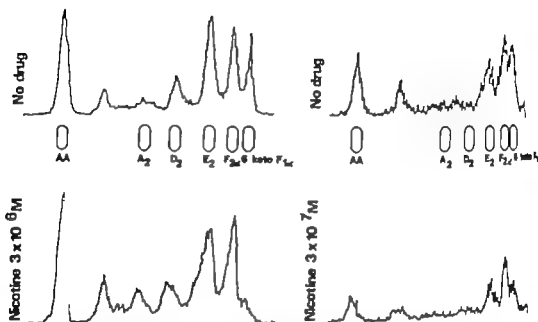
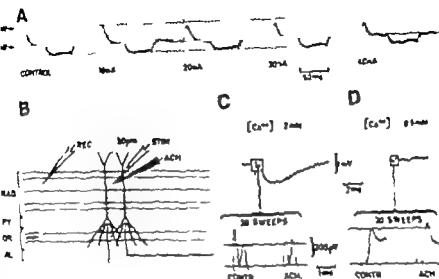
Cardiac Effluent during Infusion of ^{14}C Arachidonate

Fig. 1 *Upper panels* Radio-scans (thin-layer chromatography) of the ethyl acetate extracted cardiac effluent collected during infusion of ^{14}C -arachidonate ($0.25 \mu\text{Ci}/\text{min}$). No drug added to the perfusion solution. The radioactivity appears in three major peaks, corresponding to 6-keto-PGF $_{1\alpha}$, PGF $_{1\alpha}$, and PGE $_2$. *Lower panels* Radio-scans of the cardiac effluents as above. Nicotine, at the concentration indicated, was added to the perfusion solution 5 min before the infusion of isotope. Note that the peak corresponding to 6-keto-PGF $_{1\alpha}$ in the lower left radio-scan is almost absent. In the lower right radio-scan, the 6-keto-PGF $_{1\alpha}$ -peak is also depressed in relation to the PGF $_{1\alpha}$ -peak in comparison with the control (upper right panel).

solution appeared in three major peaks, chromatographing in parallel to 6-keto-PGF $_{1\alpha}$, PGF $_{1\alpha}$, and PGE $_2$. Sometimes there were also minor peaks in parallel to PGD and PGA (Fig. 1). The radiochromatograms from effluents collected during perfusion with nicotine containing Tyrode solution displayed a different pattern, inasmuch as the peak corresponding to 6-keto-PGF $_{1\alpha}$ was depressed (Fig. 1). The inhibition of the 6-keto-PGF $_{1\alpha}$ peak was dose-dependent, being about 60% at a nicotine concentration of $3 \cdot 10^{-7}$ M (Fig. 2).

Nicotine is probably the most common of the drugs used by man. The plasma levels of nicotine during cigarette smoking have been reported to range between 30 and 40 ng/ml (Armitage *et al.* 1974) which corresponds to the lower dose used in the present perfusion experiments ($3 \cdot 10^{-7}$ M). Thus, the possibility that tobacco smoking affects the myocardial synthesis of PGI $_2$ in man must be considered. It is well known that tobacco smoking subjects display increased morbidity in cardiovascular diseases. The present observation that nicotine inhibits the myocardial formation of 6-keto-PGF $_{1\alpha}$, precursor of the vasodilating and



1 A The amplitude of EPSPs is reduced when ACh is applied to the synaptically activated apical dendrites from Electrophoresis is performed with currents as indicated. The membrane resistance, sealed with hyperpolarized current (0.4 nA, 50 ms) and the resting membrane potential did not sag. Each trace is the average of 10 sweeps. B. Electrode arrangements used to test the excitability of axon fibers. C. Upper trace: Presynaptic volley preceding the summated EPSP. Lower trace: Higher application of the presynaptic volley. The amplitude is decreased when ACh was applied near the axon fibers. Each trace is an average of 20 sweeps. D. A C after synaptic transmission was improved by reduced Ca^{2+} concentration.

reduced to 0.5 μM (Fig. 1 D) and thus the effect was not mediated by interneurons. Atropine abolished the effect of ACh independently of the concentration of Ca^{2+} .

The experiments indicate that ACh may be a transmitter in the hippocampus of the rat both pre- and postsynaptically.

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Inhibition produced by iontophoretically applied acetylcholine in area CA1 of thin hippocampal slices from the rat

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Pyramidal cells in area CA1 of the hippocampus are excited by iontophoretically applied acetylcholine (ACh). This excitatory effect is blocked by atropine (Biscoe and Straughan 1966). Recent *in vitro* experiments suggest that the excitation is due to depolarization and is accompanied by an increase in membrane resistance (Dodd, Dingledine and Kelly 1977).

I have investigated the effect of ACh in different regions of the dendritic tree in area CA1 of the transverse hippocampal slice preparation (Skrede and Westgaard 1971; Schwartzkroin 1975). The population spike evoked by an electrical stimulus in stratum radiatum was recorded in the pyramidal cell layer. An ACh-containing glass pipette (resistance 50–80 MΩ) was used for iontophoretic application of ACh in discrete areas of the dendritic tree.

An unexpected decrease in the amplitude of the population spike was produced when ACh was applied in areas where stimulated afferents made synaptic contacts. In other areas ACh enhanced the population spike or it had no effect. Both effects of ACh were blocked when the experimental chamber was perfused with 10^{-4} M atropine added to the medium. In accordance with the report by Biscoe and Straughan (1966) pyramidal neurons, spontaneously active or driven iontophoretically by L-glutamate, were excited by ACh. It is thus unlikely that ACh produced spike inactivation as seen when L-glutamate is applied in large doses (Schwartzkroin 1975).

Intracellular recordings from pyramidal neurons showed that the depressing action of ACh was accompanied by a reduced amplitude of the evoked EPSP (Fig. 1 A). The resting membrane potential and membrane resistance were unchanged.

To investigate the possibility that the site of action was presynaptic I measured the excitability of afferent fibers in the region of ACh application (Wall 1948; Eccles 1964). The diagram in Fig. 1 B shows the electrode arrangement. The stimulus and recording electrodes were placed in the stratum radiatum 400 μ m apart, parallel to the afferent fiber direction. A presynaptic volley was recorded as a negative-positive potential preceding the EPSP from a group of nearby neurons (Fig. 1 C) (Andersen *et al.* 1977). When ACh was applied iontophoretically a few μ m from the site of stimulation the amplitude of the presynaptic volley increased. The increase was the same when the extracellular Ca^{2+} concentration was

Effects of sodium salicylate on plasma insulin concentration and fatty acid turnover in dogs

By

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Abstract

Vik Mø, H., K. Høve and O. D. Mjø. Effects of sodium salicylate on plasma insulin concentration and fatty acid turnover in dogs. *Acta physiol. scand.* 1978. 103. 113-119

The effects of intravenous sodium salicylate administration on plasma concentrations of insulin, free fatty acids (FFA) and glucose were studied in intact, nonstarved dogs both during basal and respiration-induced lipolysis. Both treatments sodium salicylate reduced the plasma concentrations of insulin. The reduction is associated with decreased plasma FFA concentrations and FFA turnover rate, while plasma glucose concentrations remained unaffected. The reduced plasma insulin concentrations effected by sodium salicylate must likely be secondary to the concomitant fall in plasma FFA concentrations due to inhibition of FFA mobilization from adipose tissue.

Key words: Fatty acids, fatty acid turnover, insulin, respiration, sodium salicylate.

New interest in the mode of action of salicylates has been created by recent findings that acetylsalicylic acid (ASA) might reduce the incidence of acute myocardial infarction in man (Boston Collaborative Drug Surveillance Group 1974, Elwood *et al.* 1974), and that ASA (Kloschies *et al.* 1975, Vik Mø and Mjø 1977) and sodium salicylate (SS) (Vik Mø and Mjø 1977) reduced the size of an acute myocardial ischemic injury in dogs. These beneficial effects have been related to the well known reduction of plasma free fatty acid (FFA) concentrations induced by salicylates (Carlson and Ostman 1961, Buzzi, Garattini and Vercoroli 1965).

The mechanism for the plasma FFA lowering effect of salicylates is not clear, however, and whether insulin is involved is not known. Although an anxiolytic effect of salicylates on adipose tissue has been demonstrated *in vivo* (Schonholzer *et al.* 1973, Stone, Brown and Steele 1969), the plasma FFA lowering effect of salicylates *in vivo* can also be due to other mechanisms. Since insulin is an important regulator of lipolysis, an elevation of plasma insulin concentration by salicylates, as reported by Field, Boyle and Renner (1967) in man, would reduce FFA mobilization from adipose tissue. On the other hand, changes in plasma concentrations of insulin might be a consequence of alterations in plasma concen-

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The effects of intravenous sodium salicylate administration on plasma concentrations of insulin, free fatty acids (FFA) and glucose were studied in intact, anesthetized dogs both during basal and norepinephrine stimulated lipolysis. In both situations sodium salicylate reduced the plasma concentrations of insulin. The reduction was associated with decreased plasma FFA concentrations and FFA turnover rate. Fasting plasma glucose concentrations remained unaltered. The reduced plasma insulin concentrations effected by sodium salicylate is most likely secondary to the concomitant fall in plasma FFA concentrations due to inhibition of FFA mobilization from adipose tissue.

Key words: Fatty acids, fatty acid turnover, insulin, norepinephrine, sodium salicylate.

New interest in the mode of action of salicylates has been created by recent findings that acetylsalicylic acid (ASA) might reduce the incidence of acute myocardial infarction in man (Boston Collaborative Drug Surveillance Group 1974, Elwood *et al.* 1974), and that ASA (Klonen *et al.* 1975, Vilk-M and Mjos 1977) and sodium salicylate (SS) (Vilk-Mo and Mjos 1977) reduced the size of an acute myocardial ischemic injury in dogs. These beneficial effects have been related to the well known reduction of plasma free fatty acid (FFA) concentrations induced by salicylates (Carlson and Ostman 1961, Bizzi, Garattini and Veneroni 1965).

The mechanism for the plasma FFA lowering effect of salicylates is not clear, however, and whether insulin is involved is not known. Although an antilipolytic effect of salicylates on adipose tissue has been demonstrated *in vitro* (Schönblatter *et al.* 1973, Stone, Brown and Steele 1969), the plasma FFA lowering effect of salicylates *in vivo* can also be due to other mechanisms. Since insulin is an important regulator of lipolysis, an elevation of plasma insulin concentration by salicylates, as reported by Field, Boyle and Remer (1967) in man, could reduce FFA mobilization from adipose tissue. On the other hand, changes in plasma concentrations of insulin might be a consequence of alterations in plasma concen-

trations of some of the substrates known to affect insulin regulation, *i.e.* glucose, amino acids, ketone bodies and FFA (Goodman 1974).

In the present study the effects of sodium salicylate infusion on plasma concentrations of insulin, glucose and FFA were investigated in order to establish possible relationships between the plasma FFA lowering effect of salicylates and the plasma insulin concentration. To obtain a wide range of FFA levels anesthetized dogs were given sodium salicylate before and during isoprenaline-stimulated lipolysis.

In addition to the possible effects of salicylates on lipolysis in adipose tissue the reduction of plasma FFA concentration induced by salicylates could be effected by an increase in tissue FFA extraction. Since salicylates *in vitro* have been reported to reduce fatty acid binding to albumin (Dawkins, McArthur and Smith 1970), a mechanism for the reduced plasma FFA concentrations might be a higher uptake of FFA in peripheral tissue at a given FFA concentration, as a consequence of lower albumin binding. A second aim of this study was therefore to examine if the plasma FFA lowering effect of sodium salicylate could alternatively be explained through a change in FFA turnover rate.

Methods

Animal preparation

Experiments were performed in 7 mongrel dogs of either sex, mean b.wt. 70 kg. After fasting overnight the dogs were anesthetized by an i.v. injection of sodium pentobarbital, 25 mg/kg b.wt. Supplemental doses were given at intervals when necessary. A femoral artery was cannulated for blood sampling and a femoral vein for infusion. Heparin, 100 I.U./kg b.wt., was given i.v. to avoid clotting of catheters.

Metabolic measurements

Arterial blood was collected into precooled heparinized plasma tubes. The blood was centrifuged immediately at 4°C, and the plasma was frozen and stored at -20°C until analysed. All analyses were done in duplicate. Plasma glucose concentration was estimated by the glucose oxidase method (Boehringer Mannheim, Germany). Plasma insulin was measured by a radioimmuno assay (Pozzanelli and Pozzanelli 1976). Bovine insulin was used both as standard and for the production of a α -trypsin antiserum. An antiserum concentration giving maximal sensitivity in the range of plasma concentrations was chosen. Assays were performed to determine whether the presence of salicylate in the assay mixture altered the binding of labelled insulin to the antisera. Within the range of salicylate concentrations found in plasma no effect on binding was observed. The concentrations of FFA in plasma were determined by the titrimetric method of Dole (1956) as modified by Trout, Estes and Friedberg (1960). The presence of salicylate in plasma slightly influenced the FFA titration, thus 67 μ mol/l per mmol/l of salicylate in plasma had to be subtracted from actually measured plasma FFA concentration.

Plasma salicylate concentration was assayed by a spectrophotometric method based on a colour reaction with ferric nitrate (Trinder 1954). Isolation of *H*-palmitic acid from the plasma was performed in a two phase extraction system as described by Kipler (1970). The radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer (Model 3320 Packard Instruments Co., Inc. Downers Grove USA).

Experimental procedure

The effects of sodium salicylate infusion on arterial plasma concentrations of insulin, FFA and glucose during basal and isoprenaline stimulated lipolysis were studied in 7 dogs. The following experimental situations were studied in each dog:

- 1) Control situation I. Immediately after the blood sampling continuous infusion of isoprenaline at a rate of 0.10–0.20 μ g/kg min.
- 2) Isoprenaline situation. Blood sampling was performed 10 minutes after the start of isoprenaline infusion.
- 3) Control situation II 30 min after cessation of the isoprenaline infusion.

→ Salicylate stimulus. Blood sampling as performed 30 min after slow i.v. injection of sodium salicylate 60 mg/kg b.w. freshly dissolved in saline and adjusted to pH 7.40, and followed by the continuous infusion of 0.15 mg/kg/min during the rest of the experiment.

→ Isoprenaline-salicylate. Blood sampling as performed 10 min after start of isoprenaline infusion in same dose as in 2).

→ 4 of the 7 expts. the effects of sodium salicylate on FFA turnover during basal and isoprenaline stimulated lipolysis were studied. H-palmitic acid with specific activity 300 $\mu\text{Ci}/\text{mmol}$ (The Radiochemicals Centre, Amersham, England) as bound to 5 bovine albumin (No. A-603, Sigma Chemical Corp. St. Louis, USA) in saline as described by Lasserri, Kayser and Carlson (1971). A continuous infusion of 1 $\mu\text{Ci}/\text{min}$ (0.5 $\mu\text{Ci}/\text{min}$) as given. The albumin-fatty acid infusion was started 90 min before the first blood sampling to give constant ^3H -palmitate acid concentration. The radioactivity in the ^3H -palmitate acid substrate as determined in duplicate samples of the infusate.

→ calculation
 → Acidic activity of plasma FFA as calculated by dividing the radioactivity of each sample by the chemically measured FFA concentration.

→ FFA turnover as calc. based from the equation

$$\frac{\text{radioactivity infusate rate (cpm/min)}}{\text{infusate FFA specific activity (cpm/mmol)}}$$

→ plasma
 → Fractional turnover of FFA (per cent/min) is calculated as

$$\frac{\text{FFA turnover}}{\text{FFA pool}} \times 100$$

→ here FFA pool = FFA concentration \times plasma volume. Plasma volume is assumed to be 5 per cent of body weight.

→ statistics
 → Wilcoxon non-parametric test for paired data (two-tailed) and Spearman rank correlation test were used to calculate probabilities (Sardorff and Cochran 1967) $P < 0.05$ regarded as not statistically significant.

Results

→ The effects of sodium salicylate on arterial concentrations of insulin, FFA and glucose before and during isoprenaline-stimulated lipolysis are presented in Table I. Plasma insulin concentration increased from 0.82 ± 0.13 to 2.87 ± 0.49 ng/ml following isoprenaline infusion (mean \pm S.E.M., $P < 0.01$). When sodium salicylate infusion was given significantly

TABLE I. Effect of sodium salicylate administration on arterial plasma concentrations of insulin, free fatty acids (FFA), glucose and salicylate before and during isoprenaline infusion in dogs. Means \pm S.E. of observations from 7 expts. are given.

Experimental situation	Insulin ng/ml	FFA $\mu\text{mol/l}$	Glucose mmol/l	Salicylate mmol/l
Control I (A)	0.82 ± 0.13	496 ± 98	6.46 ± 0.36	
Isoprenaline (B)	2.87 ± 0.49	2.157 ± 167	8.43 ± 0.78	
Control II (C)	0.43 ± 0.07	567 ± 113	6.23 ± 0.42	
Salicylate (D)	0.48 ± 0.06	411 ± 106	6.53 ± 0.27	
Isoprenaline-salicylate (E)	2.60 ± 0.30	1.439 ± 133	7.76 ± 0.65	1.19 ± 0.09
P_{A-C}		n.s.		
P_{D-D}	<0.01	<0.01	n.s.	
P_{E-E}	0.01	<0.01	n.s.	

TABLE II Metabolism of free fatty acids (FFA) during sodium salicylate administration before and during isoprenaline infusion in dogs. Mean \pm S.E. of observations from 4 expts. are given.

Experimental situation	Turnover rate of FFA $\mu\text{mol/min}$	Fractional turnover rate of FFA per cent/min
Control I	151 \pm 52	31 \pm 2
Isoprenaline	565 \pm 88	31 \pm 6
Control II	133 \pm 40	35 \pm 7
Salicylate	99 \pm 19	37 \pm 7
Isoprenaline-salicylate	352 \pm 64	28 \pm 5

lower plasma insulin concentrations were observed both before and during isoprenaline-stimulated lipolysis ($P < 0.01$).

The average plasma fatty acid concentration was increased by isoprenaline from 4% P to $2.157 \pm 0.167 \mu\text{mol/l}$ ($P < 0.01$). During sodium salicylate infusion lower FFA levels were observed both in the control situation and during isoprenaline-stimulated lipolysis ($P < 0.01$).

No significant differences in plasma glucose concentration could be detected between control situation I-II and the salicylate situation (Table I), but the concentration was increased to a similar degree both in the isoprenaline and the isoprenaline-salicylate situation.

The plasma concentrations of salicylate were 1.19 ± 0.09 and $1.08 \pm 0.08 \text{ mmol/l}$ before and during isoprenaline-stimulated lipolysis respectively.

A significant positive correlation was found between concentrations of plasma FFA and insulin ($r = 0.89$, $P < 0.01$, $n = 28$) and between glucose and insulin ($r = 0.66$, $P < 0.01$, $n = 28$) when data for basal and isoprenaline-stimulated lipolysis before and during salicylate administration were pooled.

The turnover rate of FFA was reduced by sodium salicylate in the 4 expts. measured both before and during isoprenaline infusion (Table II). The fractional turnover rates of FFA were, however, not changed by sodium salicylate.

Discussion

The main finding in the present investigation was reduced plasma insulin concentration following sodium salicylate infusion both during basal and isoprenaline-stimulated lipolysis. The insulin concentrations were positively correlated to the plasma concentrations of FFA and glucose. Since salicylate administration did not significantly influence the plasma glucose concentration either in the control situation or during isoprenaline infusion, it is unlikely that the observed effect of salicylate on insulin concentration could be mediated by a glucose action on the pancreas.

The dose of sodium salicylate in the present study was chosen so as to give a significant reduction of plasma FFA concentrations (Carlson and Østman 1961, Vik Mo and Mjøs 1976). Furthermore, the same dose of sodium salicylate or ASA effectively reduced the size of an acute myocardial ischemic injury in dogs (Vik Mo and Mjøs 1977). In man Carlson and Østman (1961) found a reduction of plasma FFA by 5 g of acetylsalicylic acid given

healthy and diabetic subjects, and recently an oral administration of about 1 g of sodium salicylate effected a marked reduction of plasma FFA in healthy fasting students (V. L. Mo, 1968 and Mjor, to be published). Thus, the dose of salicylates given to obtain an effective reduction of plasma FFA is ordinarily somewhat higher than the dose given to humans for analgesia, as also reflected in higher plasma concentrations of salicylates. Whether analgesic doses of salicylates might have some antilipolytic effect remains to be definitely established. Our finding of reduced plasma insulin concentration after sodium salicylate infusion is in accordance with Arnold and Fernstrom (1976) who found a dose dependent sodium salicylate-induced reduction in insulin concentration in rats. In contrast, salicylates caused either slight elevation (Bellefleur *et al.* 1972, Field, Boyle and Renner 1967) or no change (Limbeck *et al.* 1965) in serum insulin concentrations in humans. In these experiments, no attempt was undertaken to relate eventual changes in plasma insulin concentration to changes in plasma fatty acid concentration.

It has been shown that fatty acids play a significant role in the regulation of insulin secretion. Infusions of FFA into the pancreatic artery in dogs gave rise to a marked elevation in immunoreactive insulin in pancreatic venous blood (Crespin, Greenough and Steinberg 1973). Furthermore, elevation of plasma FFA by *ex vivo* infusion of long-chain fatty acids (Greenough, Crespin and Steinberg 1967) or by infusion of triglyceride and heparin (Madison *et al.* 1968) increased the insulin concentration in dogs. In man, administration of nicotinic acid, a potent antilipolytic agent, reduces insulin and FFA concentrations without changing plasma glucose concentration (Balasse and Ooms 1973). Since this drug has no direct inhibitory effect on pancreatic secretion of insulin (Malaisse, Malaisse-Lagae and Mayhew 1967), the observed fall in insulin concentration has been explained through reduction in FFA concentration (Balasse and Ooms 1973). The possibility that sodium salicylate might influence pancreatic secretion of insulin independently of the changes in FFA concentration, should, however be considered. Coore and Randle (1964) observed reduced insulin secretion in fragments of rabbit pancreas incubated *in vitro* with glucose when salicylate was included in the incubation medium. Seltzer (1962), however found by cannulating the pancreaticoduodenal vein in dogs no change in the release of insulin activity after salicylate administration. Although the possibility of a direct effect of salicylates on insulin secretion in dogs cannot be ruled out, the correlation between changes in FFA and insulin concentration in the present investigation makes it reasonable to assume that the lowered plasma insulin concentration observed during sodium salicylate administration was, in fact, secondary to the reduction of plasma FFA concentration. Possible functional consequences of the lowered plasma insulin concentrations effected by sodium salicylate, remains, however to be determined.

Radio-labelled palmitic acid is a suitable tracer for the study of total FFA turnover (Hagenfeldt 1975). In the present study we demonstrated that the FFA turnover rate was reduced by sodium salicylate. The fractional turnover rate, however was unchanged throughout the whole experiment, indicating that the reduced plasma FFA concentrations found after sodium salicylate administration was not due to increased uptake of FFA in the tissues. This observation is in accordance with a previous study showing that the net uptake of FFA in the myocardium of dogs was unaffected by sodium salicylate infusion during basal lipolysis.

TABLE II Metabolism of free fatty acids (FFA) during sodium salicylate administration before and during isoprenaline infusion in dogs. Mean \pm S.E. of observations from 4 expts. are given.

Experimental situation	Turnover rate of FFA $\mu\text{mol/min}$	Fractional turnover rate of FFA per cent/min
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Isoprenaline	565 \pm 88	31 \pm 6
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lower plasma insulin concentrations were observed both before and during isoprenaline stimulated lipolysis ($P < 0.01$)

The average plasma fatty acid concentration was increased by isoprenaline from 46 ± 10 to $2157 \pm 167 \mu\text{mol/l}$ ($P < 0.01$). During sodium salicylate infusion lower FFA levels were observed both in the control situation and during isoprenaline-stimulated lipolysis ($P < 0.01$).

No significant differences in plasma glucose concentration could be detected between control situation I-II and the salicylate situation (Table II), but the concentration was increased to a similar degree both in the isoprenaline and the isoprenaline-salicylate situations.

The plasma concentrations of salicylate were 119 ± 0.09 and $108 \pm 0.08 \text{ mmol/l}$ before and during isoprenaline-stimulated lipolysis respectively.

A significant positive correlation was found between concentrations of plasma FFA and insulin ($r = 0.89$, $P < 0.01$, $n = 28$) and between glucose and insulin ($r = 0.66$, $P < 0.01$, $n = 3$) when data for basal and isoprenaline-stimulated lipolysis before and during salicylate administration were pooled.

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lysis, and during isoprenaline-stimulated lipolysis net myocardial uptake of FFA was reduced by sodium salicylate (Vik Mo and Mjøs 1976)

Since salicylates have been shown to inhibit prostaglandin synthesis *in vitro* (Vane 1971) the possibility that the plasma FFA lowering effect of sodium salicylate was mediated through prostaglandins warrants consideration. The potent prostaglandin synthesis inhibitor indomethacin (Vane 1971), however, has no antilipolytic effect *in vitro* (Fredholm and Liljeqvist 1975) and furthermore, prostaglandin PGE₁ inhibits lipolysis induced by nerve stimulation in dogs (Fredholm and Rosell 1970). It is therefore unlikely that the plasma FFA lowering effect of sodium salicylate is mediated through prostaglandins.

Administration of heparin liberates the enzyme lipoprotein lipase to the circulation with hydrolysis of plasma triglycerides to FFA and heparin might therefore influence the lipometabolism. In this study however we used overnight fasted dogs in which no significant effect of heparin on arterial FFA concentrations has been observed when compared heparin doses were given (Frey 1967 Mjøs 1971).

In conclusion, intravenous sodium salicylate administration effected a reduction in plasma concentrations of FFA most likely through a non-insulin dependent inhibition of FFA mobilization. The reduced FFA mobilization by sodium salicylate seems to be due both to inhibition of adipose tissue lipolysis and to enhanced re-esterification of FFA in the same tissue (Vik Mo and Mjøs 1978).

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phonic neurons in other parts of the sympathetic nervous system. The reduction in the number of noradrenaline (NA)-containing nerves during pregnancy—with an almost complete disappearance near term followed by a restitution towards normal *post partum*—circumscribes one drastic example of the special properties of the uterine sympathetic innervation (Sjöberg 1968; Gårdmark, Öwman and Sjöberg 1971; Öwman *et al.* 1975). The changes have been followed by direct fluorescence microscopic visualization of neuronal NA in combination with chemical determinations of the total uterine level of the transmitter. The guinea-pig has for several reasons been chosen as a model for investigations on the changes underlying these and related changes (Falck *et al.* 1974; Thorbert, Alm and Norrby 1977), and the anatomy of the uterine autonomic innervation has therefore recently been described in detail in this mammalian species (Thorbert *et al.* 1977). The present study was undertaken to elucidate to which extent the fluorescence microscopic changes and alterations in the transmitter level in the uterus during pregnancy might involve descriptive phenomena, and their possible relationship to local factors associated with the young conceptus. These factors can be particularly well studied in pregnant guinea pigs, because there are often fetuses only in one horn, leaving the contralateral horn only slightly enlarged than the uterine horns of non-pregnant animals.

Materials and Methods

Animals. The material consisted of guinea-pigs (multi- or paritiparous, respectively) of several strains and housed in various reproductive series. Multiparous animals from different periods of pregnancy were selected by palpation, and after labour under light ether anaesthesia the day of pregnancy was checked by measurement of mean weight and crown-rump length of the fetuses (Draper 1970; Kamfusia 1969). The two groups were at 35–40 and 60–64 days of pregnancy. Animals from early pregnancy (18–23 days) were prepared by caesarean section, leaving a nearly broken amniotic membrane together with the male guinea-pigs for 3 days. The pregnant animals were killed 20 days later and the weight and crown-rump length of the fetuses were measured to obtain further check of the stage of pregnancy.

Paritiparous animals were also studied at 1, 4, and 12 days after delivery; the abdomen opened under ketodolac (Fluothene, ICI) anaesthesia and aseptic conditions 2–5 days *post partum* to check whether pregnancy had been unilateral or bilateral. After closure of the abdomen, 10 µg of erythrina (Erythrocytin, Fluka) as given by single intraperitoneal injection.

Non-pregnant control animals at the diestrus stage were used on day 7–12 of the estrous cycle, determined as previously described (Thorbert *et al.* 1977).

Tissue. In non-pregnant and *post partum* animals, separate tissue preparations (Fig. 1) were taken from the last 2–4 mm of the uterus adjacent to the ovary ("tubal end of the uterus"), the main part of the uterine horn, and from the cervix. The suspensory ligament, connecting the tubal end of the uterine horn to the lower rim, was also detached out. In early pregnancy (18–23 days *post partum*) and in mid-pregnancy (35–40 days), tissues were obtained from the tubal end of the uterus and the cervix as above, but the main part of the uterus was separated into the following portions (Fig. 1): the uterine horn lacking fetuses and comprising the "empty horn", the distended part of the fetus-containing horn, called the "peri-fetal uterus", and the non-distended part of the horn adjacent to (or between) the fetuses, designated the "para-fetal uterus". In pregnancy of longer duration than 45 days, each non-distended part of the uterus could no longer be distinguished (Fig. 1). Perimetrial tissue from animals at late pregnancy was studied either as whole mounts (Thorbert *et al.* 1977) or after freeze-drying and sectioning (see below).

Paracervical tissue from non-pregnant animals and at 60–64 days of pregnancy was frozen as above and sectioned (50 µm thickness) were prepared in cryostat at -23°C, dried in vacuo in desiccator over phosphorus pentoxide overnight, exposed to formaldehyde gas (see below) and further processed for fluorescence microscopy.

Uterine levels of *o*-methyl-NA. 4 animals at 60–64 days of pregnancy received 1 mg/kg of the "Tissue adrenergic transmitter" *o*-methyl-NA (Corbass, Hoechst), by single intraperitoneal injection, and were killed 15 min

Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea-pig, and their relationship to the localization of the conceptus

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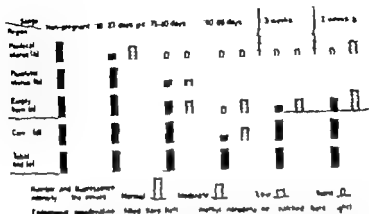
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Abstract

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Pregnancy is accompanied by a reduction in uterine noradrenaline and the study was undertaken to investigate associated structural and functional integrity of the sympathetic nerves in the organ. The formaldehyde-induced fluorescence of adrenergic nerves was studied in different uterine regions before and after in situ incubation or injection with α -methyl-noradrenaline in pregnant and puerperal guinea-pig uterus at 6 time periods, from early pregnancy (about 70 days post coitum) to 3 months post partum. The changes were related to the position of the fetuses, which were often present in only one of the two uterine horns. There was a drastic loss of fluorescent adrenergic nerves in myometrial tissue from horns devascularized by fetuses. Attempts to restore the fluorescence by incubation or injection with α -methyl-noradrenaline were essentially ineffective. Tissues from uterine regions outside (and not devascularized by) fetuses (in the case of early pregnancy), from horns devoid of fetuses (in the case of unilateral pregnancy), and from the cornu also lost their noradrenaline fluorescent nerves, but this occurred at a much later stage of pregnancy. After treatment with α -methyl noradrenaline a fluorescent plexus of sympathetic nerves could be restored to a considerable extent in these latter tissues. In puerperal animals the horn that had been devoid of fetuses regained its endogenous fluorescence much faster and the peak of α -methyl-noradrenaline as most efficient, than in the horn which had contained fetuses. In this latter horn clear signs of restoration of endogenous adrenergic fluorescence and a clear uptake capacity was not found until 3 months after delivery. In the tubal end of the uterus, the reduction in the number of fluorescent nerves was only insignificant, and the region thus clearly differed from the rest of the uterus. It is concluded that (1) there are clear regional differences with regard to the disappearance of the noradrenaline transmitter in the uterus, (2) this disappearance in early pregnancy is related to the position of the conceptus, and (3) the changes involve degenerative and regenerative phenomena as well as alterations in transmitter levels of intact neurones.

The mammalian uterus receives a well-developed supply of adrenergic nerves (Owman, Sjöberg and Sjöstrand 1974) which innervate the myometrial smooth muscle cells as well as the uterine vascular bed (Silva 1967, Marshall 1970, Hervonen and Kanerva 1973, Sporröng *et al.* 1977). These nerves differ both anatomically and functionally from the post-



2 Summary of changes in uterine adrenergic nerves in pregnant and postpartum guinea-pigs, based on fluorescence microscopic analysis described in the text. The uterus regions refer to those illustrated in 1, and the stages are expressed in terms of total post partum (p.p.) and post partum (p.p.). The tables do not take into account quantitative regional differences in the uterus (cf. Thornton *et al.* 1977) but changes in reference to the non-pregnant, ovariectomized control animal. The filled bars represent the number of fluorescent nerves containing endogenous noradrenaline, whereas the hatched bars represent the total number of green-fluorescent nerves present after incubation or injection of the (methyl)-dopamine. It should be noted that the incubation procedure results in a nerve fluorescence intensity of 100% exceeds that of the noradrenaline fluorescence found in the non-pregnant controls. Incubation experiments have usually been performed only for those at reduced number of noradrenaline-fluorescent nerves as given tables. As can be seen from Fig. 2, a peridural part of the uterus is not distinguishable and beyond late pregnancy the uterus horn then contains only of peridural tissue (cf. Fig. 1C).

other nerves, varicosities appeared somewhat swollen and the segment between the individual varicosities had little or no fluorescence. Still other fibres were not distinctly lined, suggesting a diffusion of the fluorophore. In two animals studied at the end of III stage of pregnancy there were no fluorescent terminals visible at all in the peridural area. Incubation of these slices from the peridural uterus induced a fluorescence in some fibres, but the nerve plexus was very sparse in comparison with uterine tissue from non-pregnant animals.

In the remainder of the uterine horn (between fetuses and in the tubal end), as well as in the uterine horns lacking fetuses, no certain changes could be observed in the adrenergic innervation except for a tendency to a general increase in fluorescence intensity of the nerves.

Mid pregnancy (35-40 days p.c.). At this stage the peridural uterus was completely devoid of fluorescent nerves (Fig. 3A), and in the parafetal uterus a general moderate increase in the number as well as the intensity of the fluorescent nerves had occurred (Fig. 3B). The uterine horn in these latter regions was not subject to any distension, since they are selected from uteri containing only one or two fetuses.

In the main part of the empty horn the picture of the innervation was more variable, ranging from a pattern seen in non-pregnant animals to a clear decrease in nerve fluorescence, though not to the extent seen in the parafetal uterine tissue. In the tubal end of the uterine horns whether containing fetuses or not, and in the cervix, little or no alterations could be

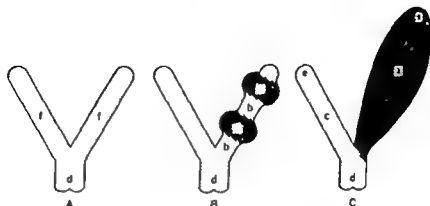


Fig. 1. Schematic representation of the guinea pig uterus showing the various regions from which preparations were taken. A, non-pregnant and *post partum* animals. B, early and mid pregnancy (late pregnancy—e, parafoetal uterus, b, parafoetal uterus, c, empty horn, d, cervix; e, total end of the uterine horn). f, main part of the uterine horn.

later for dissection of uterine tissue. The uptake of α -methyl-NA *in vitro* was studied according to the procedure described by Hamberger (1967) in preparations from 3–5 animals at the other stages. Uterine tissue slices from the main part of the uterine horns of all types (Fig. 1) were incubated for 10 min in Krebs Ringer bicarbonate buffer containing 0.1 l or 10 μ g/ml of the amine. Immediately after completed incubation, the slices were frozen in a propane/propylene mixture at the temperature of liquid nitrogen, and further processed for fluorescence histochemistry (see below). Uterine tissues from 4 non-pregnant animals treated with 0.5 mg/kg reserpine (Serpasil, Ciba) *i.p.* 24 hours beforehand (which completely abolishes fluorescence from all adrenergic nerves), were also incubated in the presence of α -methyl-NA as a control check to ascertain that a neuronal uptake did occur under the conditions used.

Histochemical procedure. Tissue pieces from the various uterine regions (Fig. 1) as well as the α -methyl-NA-incubated preparations were processed according to the fluorescence technique of Falck and Fåhr (see Björklund, Falck and Owman 1977). Formaldehyde of fixed humidity (70%) was used in order to allow a more accurate comparison of changes in fluorescence intensity. With the formaldehyde technique, the fluorophore of α -methyl-NA has the same spectral characteristics as that of NA, and the fluorescence is equal (Björklund *et al.* 1972).

Results

The organization of the uterine adrenergic innervation in non-pregnant guinea-pigs has recently been described in detail (Thorbert *et al.* 1977).

Incubation of the uterine tissue from reserpine treated non-pregnant animals (to check the reliability of the incubation technique) produced a near full restitution in the number and fluorescence intensity of the adrenergic nerves, though the axons had a more smooth (not varicosed) appearance than in preparations from non-reserpinized animals.

Changes in the histofluorescence of the adrenergic nerves in the various uterine regions at the different stages of pregnancy and *post partum* have been compiled schematically in Fig. 2.

Early pregnancy (18–23 days *p.c.*). Tissue pieces taken from the parafoetal uterus show clear fluorescence microscopic changes. In most animals, the number and fluorescence intensity of the adrenergic nerves were markedly reduced, with additional changes also in the microscopic appearance of the individual axon terminals. Sometimes the delicate fibres did not show any well-defined varicosities characteristic of the normal adrenergic nerve terminals.

In most instances the main part of the empty horn showed the same picture as the tubal end (*locus-containing*) horn, though the absence of adrenergic nerves was less obvious in the former. The tubal end of the empty horn exhibited an almost unchanged innervation compared to non-pregnant control animals.

In parametrial tissue adjacent to the uterine horns, *i.e.* the peritoneal membrane containing the primary ramifications of the uterine artery (cf. Thorbert *et al.* 1977), the adrenergic nerve terminals displayed clear reduction in fluorescence intensity and in the number per $50 \mu\text{m}^2$ of tissue.

The clusters of ganglion cells in the paracervical fibro-adipose tissue could regularly be seen. The general arrangement of these ganglion formations and of the individual ganglion cells was appreciably the same as in non-pregnant animals, though the axon bundles leaving the ganglia often tended to show an increased fluorescence intensity. Perivascular fluorescent nerve terminals were somewhat more abundant and intense than in the parametrial tissue described above.

Injection of α -methyl-NA or incubation in the presence of the amine produced principally the same results in all of the animals studied during late pregnancy. In the main part of the tubal-bearing horns, no uptake into nerve-like structures could be observed and the tissue was hence completely devoid of fluorescent nerves, whether treated with α -methyl-NA or not. However, in the empty horn, a small to moderate increase in both the number and fluorescence intensity of the adrenergic nerves occurred under the *in vivo* and *in vitro* conditions. A similar degree of increase was also seen in the cervix after injection of α -methyl-NA.

Post partum 1 and 3 weeks. In the cervix and the tubal ends of the uterine horns, a recovery of the number and fluorescence intensity of the adrenergic nerves could be recognized already 1 week *post partum*, and complete normalisation was attained within the third week. However, the main part of the horn that had contained fetuses showed no fluorescent nerves at 1 or 3 weeks after parturition. In the main part of the empty horn a clearcut return of nerve fluorescence, having an intensity resembling that of non-pregnant control animals, could be distinguished, though the number of fibres was small and variable. Characteristically some of the re-appearing fluorescent nerves differed from those seen in the uterus of the non-pregnant control animals in that many of them had a thicker and smoother appearance, and a high fluorescence intensity. The picture is interpreted as representing accumulation in the preterminal portion of the axons. Other fibres were more delicate than axon terminals in general, with varicosities of varying size and interspersed at irregular intervals.

Incubation with α -methyl-NA *in vitro* did not produce any appearance of fluorescent nerves in tissue slices from the main part of uterine horns that had contained fetuses. In the empty horn the number of fluorescent nerves seen after incubation was moderately or sometimes even markedly increased compared to corresponding non-incubated tissue. However, the density of innervation was still somewhat lower than in the uterus from non-pregnant controls.

Post partum 6 and 12 weeks. In the main part of uterine horns that had contained fetuses, the fluorescence microscopic picture was not noticeably different from that of the groups analysed at 1-3 weeks *post partum*; thus no or very few fluorescent nerves were visible. In



Fig. 3. Fluorescence photomicrograph of sections from a fetus-containing uterine horn of an animal 14 days of pregnancy. (A) Fluorescence microscopic appearance of the perifetal (distended) part of the uterine horn. (B) Para-fetal (non-distended) part of the uterine horn. That part of the uterus surrounding the fetus (A) is completely devoid of adrenergic nerves, whereas in the non-distended part from the same uterine horn, between the fetuses (B), the fluorescent nerves resemble those found in non-pregnant animals, but their number is only slightly reduced. Subtle differences in comparison with the innervation of the non-pregnant uterus include swollen varicosities and sometimes disappearance of the intervaricosities of fluorescence. $\times 120$.

detected in the number of nerves, and the fluorescence was unchanged or possibly increased.

Incubation with α -methyl-NA *in vitro* of uterine tissue from perifetal, para-fetal, or from empty horns produced no clear changes in the number of fluorescent nerves compared to non-incubated tissue. However when nerves did occur their fluorescence intensity was increased after incubation with the amine.

Late pregnancy. In the main part of fetus-bearing uterine horns there was a uniform or total lack of adrenergic nerves, irrespective of whether the tissue specimens were taken from the uterine wall underneath or far away from the placenta. A very small number of nerves with low fluorescence intensity persisted in the cervix, most often with relationship to blood vessels.

In the tubal end of the uterine horn and in the suspensory ligament, however, the fluorescence microscopic picture was clearly different, so that a great number of vascular and non-vascular nerves with a moderate fluorescence intensity still persisted. Nevertheless, the region contained a somewhat smaller number of nerves with a lower fluorescence intensity compared with non-pregnant control animals. In some preparations of the suspensory ligament there was a definite increase in the fluorescence intensity of the preterminal fibres located among the axon terminals having a reduced fluorescence intensity. Within the suspensory ligament, topographic differences were observed in the appearance of the nerve terminals. In the cranial parts of the ligament the fluorescence microscopic appearance was normal, the axons being equipped with distinct varicosities having a well-defined outline (*i.e.* without any tendency to diffusion of the fluorophore). In the caudal parts, as in the tubal end of the uterine horn, the nerve terminals had a smooth appearance. Their fluorescence intensity was lowered and they had a more diffuse contour.

as the non-pregnant weight). During early pregnancy the NA fluorescence was unchanged or, if anything, increased in this horn followed by a progressive reduction, so that no or few nerves remained visible (with a reduced intensity) at parturition and one week *postpartum*. Thereafter the innervation was greatly restored. Incubation in the presence of a methylated NA analogue resulted in a fluorescence microscopic picture indicating that at least the majority of adrenergic nerves had retained a good capacity for catecholamine uptake and accumulation. This suggests that during pregnancy the structure of the adrenergic nerve network in the empty horn has to a large extent remained intact, and that nerves have lost their NA mainly as a consequence of some humoral (local or systemic) or affecting mechanisms, such as transmitter synthesis, retention, release etc.

Perifetal uterus designates that part of the uterine horn which is located adjacent to or even fetuses, and which can be distinguished as an essentially non-distended part up to approximately 45 days of pregnancy (depending on the number of fetuses present). During time such regions could be separated from the rest of the fetus-containing horn, the fluorescence microscopic picture of the innervation followed the same pattern of changes that observed in the empty horn. There is reason to believe that they reflected altered NA uptake in an adrenergic nerve plexus where the majority—if not all—of the axons is intact.

Perifetal uterus distinguishes the uterine wall surrounding the entire conceptus, including placenta. Due to the increased space occupied by the fetus in advanced pregnancy (45 days), the perifetal uterus will later include almost the entire uterine horn except ends bordering the oviduct and cervix, respectively. Already at days 18–23 of pregnancy there was a marked reduction in the number and fluorescence intensity of adrenergic nerves in the perifetal uterus, and from 35–40 days onwards all NA fluorescence was abolished and lasted so during all subsequent time intervals studied. In no instance (except for a slight take during early pregnancy) did incubation with α -methyl-NA induce a reappearance of fluorescent nerve fibres in the perifetal myometrial slices, showing that some kind of structural change has occurred, reflected in, e.g., a reduced axonal uptake of amine. In view of previous findings of histological signs of nerve degeneration in the fetus-containing horn (Ardenmark *et al.* 1971) it is possible that these altered properties could be due to a structural change of the nerves.

Tubal end of the uterine horn, whether containing fetuses or not, showed a tendency to increased nerve fluorescence during early and mid pregnancy. Near term no clearcut increase was observed as that of the empty horn, whereas the fetus-containing horn possibly showed slight reduction in the number and intensity of the adrenergic nerves. It could not be clarified, however, to which extent the fluorescence microscopic picture reflected a decrease in the number of nerves or a dilution effect when the nerves had more altered localization in the enlarged organ. A normal fluorescence microscopic picture was seen during the *post partum* stages analyzed. Hence, the adrenergic nerves in this small region of the uterus reacted different from those in the remainder of the organ. The tubal end is a particularly well innervated part of the uterine horns receiving all of its adrenergic innervation via the suspensory ligament. This is a smooth muscle containing structure connecting the lower rib and the cranial end of the uterine horn and it contributes with as much as 35% of the total NA content in the horn (Thorbert *et al.* 1977). Thus, the adrenergic

contrast to this, incubation in the presence of α -methyl NA produced a picture different from that seen at full term and during the first weeks *post partum* (when no fluorescence could be induced). At 6 weeks *post partum* a moderate number of fluorescent nerves were seen, and at 12 weeks the density of fibres was seemingly further increased in the myometrium and around blood vessels.

The endogenous nerve fluorescence in the main part of the uterine horn that had been devoid of fetuses during pregnancy showed a high degree of recovery although a complete restoration in the number of nerves had not yet been attained at 6-12 weeks *post partum*. However, a nerve plexus of essentially the same density as in the non-pregnant animals was achieved after incubation of tissue slices from these horns in the presence of α -methyl NA.

Discussion

The level of sympathetic neurotransmitter NA in the uterus can be influenced by sex steroids (Owman and Sjöberg 1977), not only secondarily as a result of the hormone-induced changes in organ weight, but also independently in as much as the nerves seem to constitute a separate target for the action of sex steroids (Broberg *et al.* 1974; Thorbert *et al.* 1976). Induction of pseudopregnancy in rabbit is followed by a reduction in uterine NA which can be related to increased progesterone levels in both tissue and plasma (Thorbert *et al.* 1976) and reduced estradiol in plasma (Batra *et al.* 1977). Further, under the influence of oestrogenic stimulation the neuronal content of NA in the uterus increases, an effect which can be counteracted by progesterone (Falck *et al.* 1969). Also in guinea-pigs the adrenergic neurotransmitter can be changed in the uterus by sex steroids, and it has been found that combined estrogen and progesterone treatment decreases the total uterine content of NA (Thorbert *et al.* 1978). In order to relate these changes—occurring only in the adrenergic system supplying the reproductive (and perhaps also lower urinary) tract—to more physiological situations, the guinea-pig oestrous cycle (Thorbert *et al.* 1978) and pregnancy have been examined separately (Owman *et al.* 1975). It was shown that diestrous animals have higher uterine NA than oestrous ones, and it was also found that the neurotransmitter is almost entirely lost from the uterine sympathetic nerves at the end of pregnancy. In view of the above mentioned observations, together with the finding that a fall in uterine NA during pregnancy took place also in uterine horns lacking fetuses, it has been suggested that humoral factors play a role in the disappearance of uterine neurotransmitter during pregnancy (Gårdmark *et al.* 1971; Owman *et al.* 1975). Such factors might either be of systemic nature or local related to the fetus and/or placenta.

The present study has revealed a complex sequence of changes in the uterine adrenergic innervation, related not only to various stages of pregnancy but also to different regions of the uterus and the location of the conceptus. The alterations will therefore be discussed and interpreted as they have been visualized histochemically in the separate anatomical regions (Fig. 1 and 2).

Empty horn. The guinea-pig is particularly suitable for this kind of study since fetuses are often present in only one of the horns during pregnancy. The contralateral horn essentially retains its non-pregnant appearance and the increase in weight is relatively modest (14

is possible that the mechanical strain and excessive hypertrophy of the uterine wall caused with the growing conceptus are directly responsible for this nerve degeneration. However also other factors seem to have been involved because loss of NA, combined with a failing uptake mechanism, is seen also in earlier stages of pregnancy when the conceptus is still small. The same holds true for the empty horns, where the myometrial hypertrophy is relatively small and mechanical strain is insignificant. Such other factors may include acceleration or adaptive changes in those normal degenerative and regenerative rates, which have been claimed to maintain the peripheral nerve network in a state of steady turnover (Barlier and Ip 1966, Ryge *et al.* 1975). They may also include more specific hormonal changes directly related to the situation of pregnancy. The finding of a substance accumulation in otherwise only moderately fluorescent bundles of preterminals arising in the paracervical ganglia and running in the suspensory ligament, together with the swollen and distorted appearance of nerves in the myometrium, favours the view that a terminal nerve degeneration and regeneration take place in the uterus (Jorikjand and Stenerv 1973).

In the horns devoid of fetuses during (unilateral) pregnancy showed a dramatic reduction in nerve fluorescence, so that no or only few adrenergic nerves remained visible at the end of pregnancy in accordance with the course of disappearance of NA in the empty uterine horns (Owman *et al.* 1975). The rate and pattern of disappearance of fluorescent nerves differed from those in the fetus-containing horns (Owman *et al.* 1975), and by incubation with α -methyl-NA, the fluorescence could always be restored in a large number of the nerves in the empty horns. This shows that in the latter horns most of the nerves, though they had lost their endogenous NA, still retained a capacity for catecholamine uptake and accumulation. It is suggested that the transmitter in the empty horns had mainly appeared from a system of structurally intact nerves, whereas in the fetus-containing horns it was lost as a consequence of severe nerve damage and degeneration. Recent histological determinations of uterine tyrosine hydroxylase during pregnancy have shown that enzyme activity is reduced to very low values (unpublished observations) in pace with disappearance of NA from the empty horn (Owman *et al.* 1975). Since this enzyme is co-limiting in the formation of NA, impaired synthesis may be a major cause of the NA depletion in the empty horn during pregnancy. However incubations in the presence of α -methylated NA analogue did not restore an entirely normal density of the nerve fibres, which could mean that also in the empty horn nerves were damaged or degenerated, though to a much less extent than in the fetus-containing horn.

Since the empty horn undergoes relatively small changes in size, compared with the tensile enlargement of the fetus-containing horn, hormonal rather than mechanical factors seem to be responsible for the reduction in the formation and accumulation of transmitter in the adrenergic nerve plexus of the empty horn. By analogy it is conceivable that the same mechanisms underlie the NA reduction in the non-distended parts of the fetus-containing horns (paracervical uterus) during the earlier stages of pregnancy when the conceptus is still small and only partially occupies the total uterine lumen. The same reasoning also holds for the cervix region (which is common for both uterine horns), in which the pattern of changes in the sympathetic innervation both histochemically and

fibres do not originate in the paracervical ganglia, nor are they affected by transection of hypogastric nerves, in contrast to the remainder of the uterine horns (Thorbert *et al.* 1977).

Cervix. The general pattern of changes in the nerve fluorescence resembled that in the empty uterine horn. However, the fluorescence was not as markedly reduced at pregnancy and normalization after delivery was more rapid compared with the horn.

Considerations on the mechanisms of NA disappearance. The introduction of the Frit Hillarp histofluorescence technique (for references, see Björklund *et al.* 1977) has allowed a detailed mapping of the distribution of the postganglionic adrenergic neuron system in a variety of sympathetically innervated tissues and organs from numerous species. It is a general impression from this highly sensitive technique that the nerve plexus, located in the various locations, have a characteristic and constant arrangement and that the transmitter is maintained at a fairly steady level in the individual neurons within the range of physiological changes in nerve activity (Burnstock and Costa 1975). This is reflected in a rather constant NA concentration in a given sympathetically innervated organ. It is only in situations of extreme experimental conditions (e.g. Dahlström and Zetterström 1965; Nielsen and Owman 1967) or pharmacological interference with transmitter release and turnover (e.g. Malmfors 1965) that changes in endogenous NA level can be detected histochemically or chemically. The female reproductive tract appears to be unique in this sense that fluctuations in the transmitter levels can be revealed during physiological conditions such as the sexual cycle (Sjöberg *et al.* 1977; Thorbert *et al.* 1978). Such fluctuations in the transmitter content of the nerves can be mimicked by exogenous hormone treatment (Owman and Sjöberg 1977). The extremes in the physiological variability of the adrenergic innervation in the female reproductive tract are seen during the end of pregnancy when the uterus may be almost devoid of noradrenaline-containing nerves (Sjöberg 1967). The detailed changes in the NA transmitter during pregnancy as recorded by fluorescence histochemistry in the present study are in good agreement with previous chemical determinations of NA in the guinea pig uterus at various stages of pregnancy (Owman *et al.* 1976).

Silver impregnation of tissue preparations taken from fetus-containing uterine horns at late pregnancy in guinea pigs showed a reduced number of nerve fibres (compared with empty horns), and several of the nerves showed signs of degeneration with a swollen appearance or were granulated and had disintegrated into small fragments (Gårdmark *et al.* 1971). It is thus possible that the disappearance of NA-containing nerves from the fetus-containing horns is at least partly due simply to loss of nerve structure. This would agree with the inability to regain nerve fluorescence after incubation in the presence of a methyl-NA. In control experiments with reserpine in which the NA had been depleted from the non-pregnant uterine tissue, a near complete restoration of the number of visible fluorescent nerves was obtained, showing that the incubating technique adequately demonstrated the presence of NA-deficient nerves with a functioning axonal membrane potential. There is evidence from silver impregnation studies (Gårdmark *et al.* 1971) that the fetus-containing horn at late pregnancy also contains structurally more intact nerve fibres, but apparently with a functional damage as reflected in the defective uptake capacity. It can be assumed that this severe impairment represents one stage in a degeneration process.

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chemically (Owman *et al* 1975) followed those in the empty horn of the uterus (tail parafoetal uterus). This humoral influence would be in accordance with the sensitive uterine adrenergic nerves to the action of sex steroids as evidenced by reductions in NA levels during HCG induced pseudopregnancy (Thorbert *et al* 1976), oophorectomy (Falk *et al* 1974) or treatment with estrogen and progesterone (Falk *et al* 1969 Thorbert 1978).

The fluorescent nerve plexus in the uterine horn that had been devoid of NA, restored to a great extent within three weeks after delivery whereas no endogenous fluorescence was visible in the other horns. Even after twelve weeks only a small number of nerves were visible in the latter horns, though a nerve fluorescence could be induced by incubation with α methyl NA. It is assumed that there is a "refilling" of NA in the nerves in the empty horns, whereas the late reappearance of adrenergic fibres in the horns that contained fetuses mainly involves a regenerative phenomenon. Not until approximately twelve weeks *post partum* is a nerve plexus restored, but it still lacks the capacity to accumulate sufficient amounts of endogenous NA for fluorescence histochemical visualization. The time-course of reappearance of these nerves is in good agreement with that after treatment with 6-hydroxydopamine or antibodies to Nerve Growth Factor (Ruitink *et al* 1975). It should be mentioned that previously reported figures on the reappearance of uterine NA after delivery (Gårdmark *et al* 1971) are based on determinations which included also the cervix (and the tubal end of the uterine horn) where the NA restoration is rapid. The NA values given are therefore higher than would be anticipated from the present fluorescence histochemical analysis of the main part of the uterine horns during the *post partum* period.

From earlier studies and the present fluorescence histochemical findings on the changes in the neuronal NA and the capacity of the uterine adrenergic nerves to accumulate exogenous transmitter analogue II can be concluded (a) that there are clear regional differences in the disappearance of uterine NA, (b) that this disappearance is related to position of the conceptus, and (c) that the changes involve de- and regeneration of nerves together with alterations in the NA level of intact nerve fibres.

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Three types of motor units with different functional properties have been identified in the inferior oblique muscle (Lennnerstrand 1974, 1975). The units with the fastest con- traction are probably composed of singly innervated fibres and the slowest units of multiply innervated fibres of the slow amphibian type. The units of intermediate speed of contraction have been suggested to consist of multiply innervated fibres resembling the slow fibres in the cat muscle (Bach-y-Rita and Ito 1966, Lennnerstrand 1974, 1975, Lennnerstrand and Bach-y-Rita 1975). It is not known how the properties of eye muscle fibres in the cat change from birth to the adult stage. Matyushkin (1967) reported that the contraction of eye muscles in young rabbits were slower than those of adult muscle, and similar observations have been made on rat eye muscle by Close (1975).

In recent years the postnatal development of skeletal muscle in the cat has been extensively studied, particularly the functional differentiation of the fast and slow twitch fibre systems in limb muscles (Buller, Eccles and Eccles 1960, Buller and Lewis 1965, Close and Hoh 1967, Mann and Salatsky 1970, Bagust, Lewis and Westerman 1973, Westerman *et al.* 1973, Hammarberg and Kellerth 1975 a, b). At birth all hindlimb muscles contract slowly. They are to be composed of the same types of fibres (Nyström 1968 b, Maier and Eldred 1974, Hammarberg 1974). During the first postnatal weeks a differentiation takes place and some muscles like the gastrocnemius increase their speed of contraction with age, while others like the soleus remain slow throughout development (Buller *et al.* 1960, Buller and Lewis 1965, Nyström 1968 a, Westerman *et al.* 1973). The differentiation of fast and slow contraction seems to occur in parallel with changes in fibre composition (Nyström 1968 b, Maier and Eldred 1974, Hammarberg 1974).

The purpose of the present investigation has been to study the postnatal development of the extraocular muscle with physiological and histochemical techniques. A comparison with the development of fast and slow fibre systems of other cat muscles will be attempted although this will be somewhat difficult due to the lack of information on the postnatal development of muscle fibres with multiple innervation, even in amphibian and avian muscle. Isometric contractions have been recorded in the inferior oblique muscle of kittens of different ages and in the adult cat. It has not been possible to record from individual fibres or motor units, and conclusions have to be drawn from data obtained from the whole muscle. In this paper the postnatal changes in twitch and tetanic contraction will be evaluated. In a subsequent paper the changes with age in fatigue properties and other post-tetanic effects will be examined (Lennnerstrand and Hanson 1978). The developmental changes in the histochemical fibre spectrum of the inferior oblique muscle will be described separately (Hanson, Nichols and Lennnerstrand 1978). Preliminary reports on the results have been published elsewhere (Hanson, Lennnerstrand and Nichols 1977, Lennnerstrand, Hanson and Nichols 1977).

Methods

25 kittens and 3 adult cats were used in the study. They were arranged in 8 groups according to postnatal age. The number of animals in each group and their body weight are shown in Table 1. Most animals of both eyes were used for experiments.

The animals were anaesthetized with pentobarbital (Nembutal®) 40 mg/kg b.w. injected intraperitoneally and the trachea and one femoral vein were cannulated. Additional doses of anaesthetic were given if needed. The head was rigidly clamped with suryette and bite bar. The inferior oblique, the lateral and

The postnatal development of the inferior oblique muscle of the cat

I. Isometric twitch and tetanic properties

By

GUNNAR LENNERSTRAND and JERKER HANSON

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Abstract

LENNERSTRAND G and HANSON J *The postnatal development of the inferior oblique muscle of the cat. I. Isometric twitch and tetanic properties* Acta physiol. scand. 1978; 103: 132-143.

The postnatal development of the inferior oblique muscle in the cat has been studied with physiological and histochemical techniques. This paper describes the changes with age in isometric twitch and tetanic response characteristics. The twitch amplitude increased and the twitch contraction time (cr) and relaxation time (lrr) decreased almost linearly from birth to adulthood. The relation between the area of nerve stimulation and twitch cr and lrr changed during development with a threshold value appearing at 10 weeks. Twitch responses in cats 6 weeks of age or older were of longer duration than in younger cats, in spite of the longer cr and lrr in young cats. Fusion frequency of the tetanic response was a constant level in muscles 6 weeks or older. The maximum rate of tension rise remained the same from 6 to 6 weeks of age and later increased markedly up to 20 weeks of age. The contracture induced by tetracycline was the same in muscles of all ages. These data were related to previous findings on the postnatal development of fast and slow muscles and motor units in the hindlimb of the cat. A difference in the development of fast and slow eye muscle fibres is suggested. Slow fibres seemed to have completed their maturation at about ten weeks of age, while the development of fast fibre properties continued probably up to the adult stage.

Key words: Postnatal development, cat extraocular muscle, fast and slow fibres, contractile properties, twitch and tetanus.

With regard to muscle fibre structure, adult eye muscle is known to differ markedly from other muscles. The inferior oblique muscle of the cat has been shown to contain slowly (focally) innervated muscle fibres of the same types that exist in mammalian skeletal muscle (Alvarado and van Horn 1975), but in addition multiply innervated fibres, not seen in ordinary skeletal muscle, have been identified (Hess and Pilar 1963, Peachey, Takeuchi and Nag 1975, Alvarado and van Horn 1975, Mayr 1975). The multiply innervated fibres consist of 2 types, one of them resembling slow fibres in amphibian muscle and the other slow fibres in avian muscle (Peachey 1971, Peachey *et al.* 1975, Alvarado and van Horn 1975, Mayr 1975).

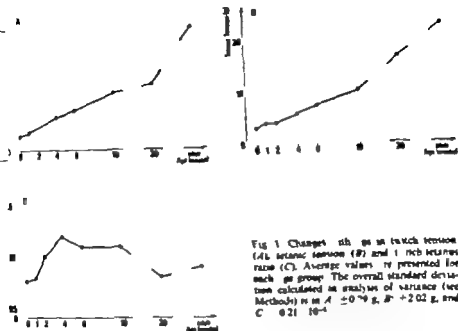


Fig. 1. Changes with age in twitch tension (A, B) and twitch/tetanus ratio (C). Average values are presented for each age group. The overall standard deviation calculated in analysis of variance (see Methods) is in A ± 0.79 g, B ± 2.02 g, and C $\pm 0.21 \times 10^{-1}$.

tetanus tension increased with age in about the same manner. At all ages the maximum tetanic tension was obtained at stimulus rates that produced a smooth tension curve, i.e. the fusion frequency. This is well known from previous studies of adult cat eye muscle (Jernöck, Bell and Rausch 1971; Lennestrand 1974), and seems to hold also for developing eye muscles.

Twitch/tetanus ratio. The mean twitch/tetanus ratios for each age group are shown in Fig. 1 C. The values were low at birth. They shifted to a higher level in animals between 4 and 10 weeks of age but became low again in the adult cat. These age variations were statistically significant at the 5% level.

Twitch contraction. Contraction times (ct) and half-relaxation times (hrt) of the twitch have been plotted against age in Fig. 2 A and B. For comparison, mean values of the same parameters during development of the gastrocnemius and soleus muscles, obtained from the work by Hammarberg and Kellerth (1975 a), have been included in the graphs. Disregarding the fact that the ct and hrt values were much lower for the inferior oblique muscle than for the hindlimb muscles, it was found that the postnatal changes in ct and hrt were qualitatively similar in the inferior oblique and the fast gastrocnemius muscles, except at the ages above 10 weeks (Fig. 2 A, B). The ct and hrt at birth were approximately twice as large as the values obtained in the adult animal. In the inferior oblique the ct and the hrt continued to decrease beyond 20 weeks of age while the development with respect to speed of twitch contraction was completed within 6 weeks of age in the gastrocnemius muscles.

As seen in Fig. 2 A the reduction in ct of the inferior oblique muscle was more or less linear with age. The decline of the hrt (Fig. 2 B) was slower in the age period between 6 and 20

TABLE 1 Presentation of the groups of experimental animals in the study

Group	Age (days)	Weight (g)	Animals	Muscles
0 week	2-3	138-143	3	6
1 week	6-9	195-261	4	7
2 weeks	12-17	221-326	4	8
4 weeks	29-32	308-487	5	9
6 weeks	42-45	348-550	4	7
10 weeks	66-74	600-900	3	6
70 weeks	123-151	650-1200	2	4
Adult	over 365	800-3500	3	5

medical recti and the retractor bulbi muscles were freed from the globe, which was eviscerated. The tilting membrane was raised to form a pool in the orbit, which was filled with warm mineral oil (37°C). The body temperature was monitored continuously and regulated to 37-38°C with a heating pad. At the experiments parts of the muscles were removed for histochemistry.

Isometric tension recording. The short tendon of the inferior oblique muscle was tied with braided silk to a hook, glued to one end of a sensitive strain gauge (Endevco no. 8101). The other end of the strain gauge was clamped to a metal base attached to a micromanipulator. The undamped resonance frequency of the tension recording assembly was 2 kHz. The linear range of the system spanned from 10 mg to 4 g. The signals from the strain gauge were amplified and displayed on a Medelec UV recording unit. The pass band of the amplifiers was set at 0-10 kHz. In order to enable comparison between contractions of different muscles, all tension recordings were made at the muscle length of maximal twitch response. It was taken to align the strain gauge perpendicular to the natural pulling direction of the muscle.

Stimulation. The muscle nerve was carefully dissected free from orbital tissue and cut as far from the muscle as possible. It was positioned over paired platinum electrodes and stimulated with square-wave pulses of 0.1 ms duration, generated by a Grass S8 stimulator and fed through an isolation unit.

Recording and measurements. With some slight modifications the techniques have been similar to those used by Hanson and Linnérstrand (1977). *Twitch responses* were recorded first. Increasing increasing single pulse stimulation from threshold to supramaximal values. Threshold was about 0.4 V for the newborn animals and decreased to about 0.2 V for adult cats. Stimulus intensities at least five times the maximal twitch response were used for the rest of the recordings of twitch and tetanic responses. Maximal amplitude of the twitch, the contraction time (t_c) and the half-relaxation time ($t_{1/2}$) of the twitch were measured. In addition the time for complete relaxation of the twitch was recorded as described in Results. *Tetanic responses* were elicited by trains of pulses ranging in frequency from 5 to 800 Hz. The *fusion frequency* at which the tetanic stimulation was determined, i.e. the lowest stimulus frequency to obtain a totally fused tetanic output with the present recording system. The minimum level of detection of the sensitive tension device was just below 10 mg, which was sufficient for the determination of frequency even in the newborn muscles, with tetanus tension of 1-3 g. The *maximum tetanic tension* and the *rate of tension rise* was measured for different stimulus frequencies. The rate of tension rise is taken as the steepest slope of the rise as part of tetanic response. The values were expressed as the tension change per cent of the maximum tetanic tension per millisecond. *Saccharin* (Sch) was injected i.v. in a dose of 1 mg per kg b.w. and the contracture induced in the inferior oblique by this drug was recorded. Animals were ventilated on pump during the transient paralysis of the respiratory muscles.

Statistical analysis. When both muscles of the same animal were used the values were lumped. An analysis of variance (one way) was performed for each parameter studied in order to reveal significant differences between any of the age groups. For more detailed comparison between age groups, confidence intervals were calculated according to the method of multiple comparisons of Scheffé (1961). In all graphic presentations of results mean values have been plotted. The standard deviations for each parameter were of the same order of magnitude in all age groups, and could therefore be represented by the over all standard deviation from the analysis of variance. This is given in the legend to each diagram.

Results

Twitch and tetanic tension. Values for maximum twitch and tetanus tension produced by the inferior oblique muscle at various ages are given in Fig. 1 A and B, respectively. Twitch and tetanic

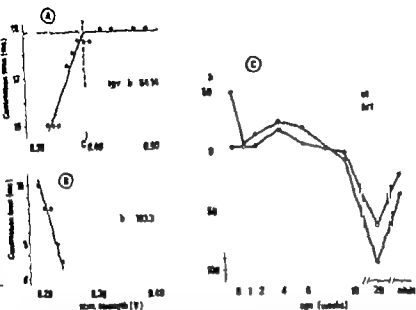


Fig. 3. Changes in cr and lrt with stimulus intensity of nerve stimulation. A shows the initial increase with stimulus intensity in muscle from newborn kittens and B the decrease in cr in the case of old animals. All regression lines represent stimulus intensities cr levelled off. The parameters a , b and c from equation in the text are marked. These show that the regression lines calculated on cr and lrt are the "method of least squares". In C mean slope values (b) for relations between stimulus intensity and cr and lrt have been plotted for all age groups. The overall standard deviation is ± 4.4 for cr and ± 10.3 for lrt. The graphs indicate that young animals have positive slope and older animals a negative slope. Difference between age groups is statistically significant only for cr .

ages. The time for total twitch tension decline was estimated from double twitch responses stimulation at low repetition rates (10 Hz and below see also Hanson and Lennestrand 1971). At very low stimulus frequencies the first twitch had completely vanished before the pulse to the second stimulus appeared. The lowest frequency at which the two twitches tried to show summation has been plotted in Fig. 3 B for muscles of different ages. The ages for the newborn cat were the highest. Thus, in spite of the long lrt at this age, the fall of the twitch response was faster than in older muscles. The difference between born and 6 weeks old animals was statistically significant ($p < 0.01$).

Twitch contraction and stimulus strength. On increasing the intensity of nerve stimulation to threshold to supramaximal values, more and more motor units were recruited in the muscle, which led to an increase in twitch amplitude and to changes in cr and lrt. The cr and lrt were studied in some detail, since it has been shown previously that cr and lrt of adult eye muscle, in contrast to other skeletal muscle, had higher values at threshold than at maximal stimulus intensities (Lennestrand and Bach-y-Rita 1974). This means that cr and lrt decreased with increasing stimulus strength in the lowest intensity range. Later as the maximal response level was approached, the twitch cr and lrt increased with stimulus intensity. The youngest muscles, on the other hand, showed steady increase in both cr and

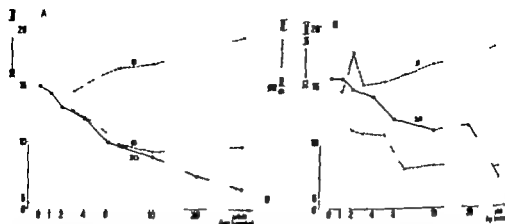


Fig. 2. Twitch contraction time (*ct*) in A and half-relaxation time (*hrt*) in B plotted against age in inferior oblique (IO), the gastrocnemius (G) and soleus (S) muscles. The values of G and S obtained the work by Hammarberg and Kellerth (1975) (courtesy of Dr J.-O. Kellerth). Note, that the left scale pertains to IO readings and the right scale to G and S values.

weeks than at earlier and later ages, but the difference in *ct* and *hrt* development was statistically significant. This was tested by calculating the *ct/hrt* ratio for each muscle and comparing the values of the different age groups.

Twitch duration In Fig. 3 A, showing twitch responses from kitten eye muscles of 0 and 6 weeks and from an adult muscle, it can be seen that total twitch duration was shorter in the youngest than in the older muscles. Since the *hrt* was longer the younger the muscle (Fig. 2 B) it must be the latest phase of the twitch that tailed off more slowly in the old

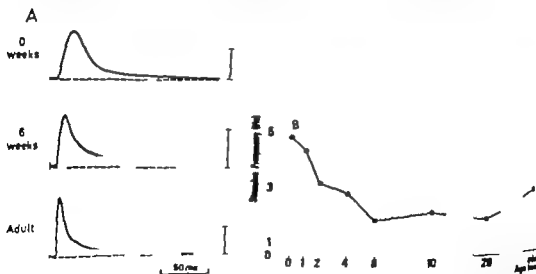
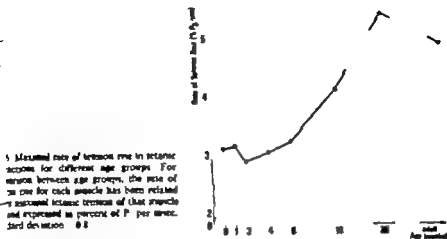


Fig. 3 A. Twitch responses obtained in three different inferior oblique muscles of ages marked. Broken line indicates resting tension before stimulation. The decline of the twitch response is of longer duration in the 6 weeks old animal and the adult cat than in the new-born animal. Bars to the right of traces indicate stimulus frequency: 0.2 g. and in middle and bottom panels 0.5 g. Same time scale in all recordings. B. Twitch duration estimated from the lowest stimulus frequency to produce summation between the twitches in double stimulation. Note, that stimulus frequency was higher and twitch duration thus shorter in young than in old kittens. Average values given for each age group. S.D. ± 0.9 Hz.



isotonic values for cr and hcr at threshold and at maximal activation are given in Table II. It was found that the threshold cr but not hcr increased continuously with age. Fusion frequency. As shown in Fig. 5 the fusion frequency was around 700 Hz in the newborn kitten and increased with age until the values saturated at about 325 Hz for muscles over 6 weeks. The difference between values above 6 weeks of age and those below 2 weeks was statistically significant ($p < 0.01$).

Maximal rate of tension rise. Stimulus frequencies higher than the fusion frequency had to be applied in all age groups in order to attain maximal rate of rise of the tetanic response. The newborn animal stimulation at 400 Hz was necessary and in the adult cat 600 Hz. The values of rate of tension rise in Fig. 6 have been expressed as the tension increase in percent of maximal tetanic tension (P_0) per millisecond. There was no significant variation between muscles up to 6 weeks of age. At 10 weeks the values started to increase and at 20 weeks they were even higher than in the adult animal.

Effect of succinylcholine (Scl). This drug is known to cause a long-lasting contracture in trochlear muscle of the adult cat, probably by inducing an extensive depolarization of the

Table III Effects of succinylcholine on inferior oblique muscles at different ages. Drug action has been expressed as succinylcholine-induced tension in percent of the maximal tetanic tension, and has been measured at the time of maximal succinylcholine effect and 5 and 10 min later. Mean values (\pm S.E.) are given for each age group.

Age	Max. effect	5 min	10 min
newb.	46 (\pm 9)	12 (\pm 6)	17 (\pm 16)
week	44 (\pm 11)	6 (\pm 6)	1 (\pm 1)
weeks	33 (\pm 7)	7 (\pm 5)	3 (\pm 3)
1 weeks	33 (\pm 13)	15 (\pm 11)	5 (\pm 4)
weeks	48 (\pm 11)	18 (\pm 9)	4 (\pm 3)
2 weeks	46 (\pm 6)	3 (\pm 5)	1 (\pm 1)
3 weeks	41 (\pm 3)	21 (\pm 2)	14 (\pm 2)
4 weeks	44 (\pm 6)	21 (\pm 6)	13 (\pm 6)

TABLE II Twitch cr and Art obtained with threshold and supramaximal stimulation, respectively in eye muscle of different age. Mean values (\pm S.D.) are given.

Age	cr		Art	
	Threshold	Supramax.	Threshold	Supramax.
0 week	12.2 (\pm 2.0)	15.3 (\pm 2.9)	13.5 (\pm 3.7)	16.0 (\pm 3.0)
1 week	11.6 (\pm 1.3)	14.4 (\pm 0.8)	13.1 (\pm 1.5)	15.3 (\pm 4.9)
2 weeks	9.9 (\pm 1.7)	13.1 (\pm 0.8)	11.4 (\pm 1.8)	14.8 (\pm 2.7)
4 weeks	9.1 (\pm 2.3)	12.0 (\pm 0.9)	12.0 (\pm 2.5)	13.0 (\pm 1.5)
6 weeks	8.4 (\pm 1.2)	10.0 (\pm 0.8)	11.6 (\pm 2.0)	12.0 (\pm 1.0)
10 weeks	10.5 (\pm 2.4)	9.0 (\pm 1.5)	13.3 (\pm 3.7)	11.0 (\pm 1.5)
20 weeks	8.6 (\pm 2.5)	7.0 (\pm 0.21)	15.3 (\pm 7.0)	10.8 (\pm 2.1)
Adult	7.8 (\pm 1.6)	6.0 (\pm 0.8)	11.0 (\pm 1.4)	7.4 (\pm 1.6)

Art with increasing stimulus strength as exemplified in Fig. 4 A for twitch cr . A change in the adult pattern was observed in kittens 10–20 weeks of age (Fig. 4 B).

In order to compare twitch parameters in muscles of different age, the relation between the stimulus intensity and cr or Art was approximated from the graphs as shown in Fig. 4 and expressed in following

$$y = \begin{cases} a + bx & \text{if } x < c \\ a & \text{if } x > c \end{cases}$$

where x is threshold intensity, y intensity of reach maximal response level a , and b the slope of the line (see Fig. 4 A). A positive slope denotes an increase in cr or Art with stimulus strength and a negative slope the reverse. An analysis of variance and calculation of confidence intervals according to the method of Scheffé (1951) was performed for the b -values of muscles from cats of different age.

Significant differences were revealed for cr of kittens below ten weeks of age and for adult cats. However, there were no statistical difference between any age groups with respect to Art changes with stimulus intensity although the mean values of cr and Art seem to change in a similar way with age (Fig. 4 C). The standard deviation for Art was much larger than for cr . These findings strongly support the idea that the recruitment of motor units to increase stimulus intensity followed different patterns in young and in adult eye muscles, with a period of transition at 6–10 weeks.

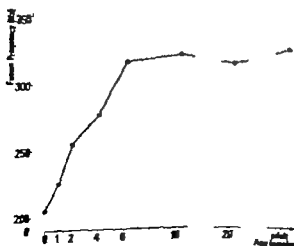
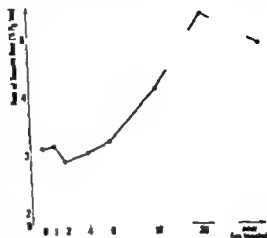


Fig. 5 Changes in fusion frequency of eye contraction with age. A cage shows plus standard deviation \pm 27.5 Hz.



Maximal rate of tension rise in tetanic contractions for different age groups. For tension between age groups, the rate of rise for each muscle has been related to maximal tetanic tension of that muscle and expressed as percent of P per msec. and deviation 0.8 %.

threshold values for cr and lcr at threshold and at maximal activation are given in Table II as found that the threshold cr but not lcr increased continuously with age.

fusion frequency As shown in Fig. 5 the fusion frequency was around 200 Hz in the born kitten and increased with age until the values saturated at about 3.5 Hz for muscles cr then 6 weeks. The difference between values above 6 weeks of age and those below 2 weeks was statistically significant ($p < 0.01$).

Maximal rate of tension rise Stimulation frequencies higher than the fusion frequency had to be applied in all age groups in order to attain maximal rate of rise of the tetanic response. In the newborn animal stimulation at 400 Hz was necessary and in the adult cat 600 Hz. The values of rate of tension rise in Fig. 6 have been expressed as the tension increase in percent of maximal tetanic tension (P_0) per millisecond. There was no significant variation between muscles up to 6 weeks of age. At 10 weeks the values started to increase and at 16 weeks they were even higher than in the adult animal.

Effect of succinylcholine (SCh). This drug is known to cause a long-lasting contracture in skeletal muscles of the adult cat, probably by inducing an extensive depolarization of the

TABLE II Effects of succinylcholine on inferior oblique muscles of different ages. Drug action has been expressed as succinylcholine-induced tension in percent of the maximal tetanic tension, and has been measured at the time of maximal succinylcholine effect and 5 and 10 min later. Mean values (\pm SD) are given for each age group.

Age	Max. effect	5 min	10 min
0 week	46 (\pm 9)	12 (\pm 6)	17 (\pm 16)
1 week	44 (\pm 11)	6 (\pm 6)	1 (\pm 3)
2 weeks	38 (\pm 7)	7 (\pm 5)	3 (\pm 3)
4 weeks	41 (\pm 13)	15 (\pm 11)	5 (\pm 4)
6 weeks	46 (\pm 11)	15 (\pm 9)	4 (\pm 5)
8 weeks	46 (\pm 6)	5 (\pm 5)	1 (\pm 1)
10 weeks	41 (\pm 11)	21 (\pm 23)	14 (\pm 23)
Adult	44 (\pm 6)	21 (\pm 6)	13 (\pm 6)

multiply innervated fibres (Bach-y Rita 1971). A contracture could be elicited by in kitten inferior oblique muscle of all ages. Drug action was expressed as the % tension in percent of the maximum tetanic tension. There was no significant difference in drug action between age groups (Table III). The drug effects 5 and 10 min after were slightly larger in the older animals. Duration of drug action was 10 to 15 min in at age 10 weeks and below and slightly longer (15–20 min) in the older animals.

Discussion

Postnatal differentiation of fast and slow fibres

At birth the inferior oblique was a much slower muscle than in the adult cat. The speed of twitch contraction increased gradually over at least the first half year of the kitten's life. In this respect the development of eye muscles was similar to that of fast limb muscles although in the latter adult values were already obtained at the age of 2 months (Quarles *et al.* 1960; Buller and Lewis 1965; Mann and Salafsky 1970; Westerman *et al.* 1973; Farnham and Kellerth 1975 a). However if speed of contraction in eye muscles is estimated from the stimulus frequency to produce a fused response, the mature stage seems to be reached by 6 weeks. By using the rate of tension rise as the measure of contraction speed, one would conclude that muscle development did not start until about 6 weeks of age and continued to approximately age 20 weeks.

The differences between the postnatal development of fusion frequency and twitch contraction time might be related to post-tetanic effects. The twitch responses reported in this paper were not influenced by previous muscle activity. It will be shown in a subsequent paper (Lennerstrand and Hanson 1978) that repetitive stimulation prolonged twitch relaxation and induced a staircase phenomenon in the tetanic response. These effects were more marked in the older animals, and could account for most of the levelling off of the curve relating fusion frequency to age.

In order to explain the developmental changes in the rate of tension rise of eye muscles one might propose that the slow fibres matured more rapidly than fast fibres. Constant values for the rate of tension rise during the first 6 weeks in spite of a decrease in twitch contraction time would indicate that the slow fibre system completed most of its development during this period. The parallel changes in twitch contraction time and rate of tension rise up to age 20 weeks suggest that the fast fibres obtain their adult properties much later.

Another finding to support the idea that the development of slow and fast fibres of eye muscles followed different time courses was that the time for total twitch relaxation decreased from birth to six weeks of age, which would indicate that the slowest fibres, which contribute most to the latest phases of the twitch response, not only remained slow from birth but became still slower with age.

The eye muscle responses to threshold nerve stimulation

It was found that increasing the intensity of nerve stimulation from the threshold to a supramaximal level affected twitch responses differently depending on the age of the animal. In the young muscles, fast fibres seemed to be activated first and

and more slow fibres were recruited. The same pattern is seen in ordinary skeletal muscle irrespective of age. However in cat eye muscle of age 10 weeks the response to threshold stimulation is slower than those to supra threshold stimulus intensities. This pattern is regularly seen in the adult muscle (Lennnerstrand and Bach-y-Rita 1974). The slow fibres responding at threshold must be innervated by large diameter nerve fibres, as these could be activated by the lowest stimulus. It seems unlikely that the muscle in question would be innervated by small nerve fibres in the young animal and later innervated by large nerve fibres, or that the nerve fibres to these muscle fibres should be grown at a faster rate than other nerve fibres. Fast hindlimb muscles are known to develop larger nerve fibres than slow muscle (Nyström 1968 b), as reflected also in the higher conduction velocities of nerve axons innervating fast motor units than slow motor units (Agost *et al.* 1973, Bagust 1974). This relation holds also for kitten hindlimb muscle (Bagust, Lewis and Westerman 1974). Since the number of muscle fibres seems to remain unchanged during postnatal development (Westerman *et al.* 1973), the most plausible explanation for the appearance in eye muscles of a slow response to threshold nerve stimulation at 10 weeks age would seem to be that some portion of the muscle fibres had changed their twitch properties and developed slightly slower contractions, in the same manner as the slow fibres of the soleus muscle (*cf.* Fig. A and B). These fibres may be located in the outer orbital per se since findings in the adult cat indicate that the motor response to threshold stimulation was in orbital fibres (Lennnerstrand and Bach-y-Rita 1974, Bach-y-Rita 1975). The histological study of postnatal changes in eye muscles (Flanston *et al.* 1977) has shown that the third layer is poorly defined at birth but fully developed at about 10 weeks of age.

Effects of succinylcholine

The succinylcholine experiments gave similar results at all stages of development. In response to an injection of the same amount of the substance per unit kg wt. , the muscles developed contractions that amounted to 40–50% of the maximal tetanic tension. The drug is thought to induce a long-lasting contraction of multiply innervated muscle fibres, *i.e.* the slow fibre components, by depolarizing the postsynaptic region of the muscle fibre membrane (Bach-y-Rita 1971). In eye muscle of the adult cat the drug would seem to act on multiply innervated fibres of both the sphincter and the saccular types (Bach-y-Rita *et al.* 1977). The multiple innervation of adult extraocular muscle is probably only to a very small extent of the polysynaptic type, with overlapping innervation from several nerve fibres to the same muscle fibre (Bach-y-Rita and Lennnerstrand 1974). It is well-known, however that all fibres in the hindlimb muscle are polynuronally innervated at birth, but that this type of innervation has disappeared at 11 weeks of age (Bagust, Lewis and Westerman 1973). The possibility of polynuronous innervation of eye muscle fibres in young kittens makes it difficult to draw conclusions on the development of multiply innervated fibres on the basis of the succinylcholine experiments.

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multiply innervated fibres (Bach-y Rita 1971). A contracture could be elicited by the In kitten inferior oblique muscle of all ages. Drug action was expressed as the Schöten tension in percent of the maximum tetanic tension. There was no significant difference in drug action between age groups (Table III). The drug effects 5 and 10 min after the injection were slightly larger in the older animals. Duration of drug action was 10 to 15 min in most at age 10 weeks and below and slightly longer (15–20 min) in the older animals.

Discussion

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Each muscle declined with adult patterns were reached at about 6 weeks (Hanssonberg and Kelferth 1975 a). The motor units of slow twitch muscle remained moderately or highly resistant throughout development. In fast twitch muscle fatigue resistance decreased in units that developed into fast units but remained high in the units that became slow (Hanssonberg and Kelferth 1975 c).

Post-tetanic potentiation (PTP) has not been systematically studied in adult eye muscles during postnatal development. In hindlimb muscles PTP is known to be more pronounced in slow twitch than in fast twitch muscle at birth, the conditions reverse in adult animals, i.e. clear PTP in the fast muscles and almost none in the slow ones (Standæert 1964, Nyström 1968). With regard to post-tetanic effects in individual motor units of adult hindlimb muscles, Olsson and Swett (1971) reported that slow (S) units and fast, quickly fatiguing (F) units show very little PTP but that PTP was prominent in fast, fatigue resistant (FR) units. PTP in motor units during postnatal development has not yet been thoroughly examined.

In view of the differences between slow and fast fibres in other muscles, a study of fatigue in PTP might give further insight into the development of fast and slow components in eye muscles. A preliminary report on the results has been published elsewhere (Hansson, Lennernäs and Nicholson 1977).

Methods

General procedures used for activation of the inferior oblique muscle in kittens of various ages and for recording of isometric tension responses have already been described (Lennernäs and Hansson 1978). In this paper post-tetanic properties were studied in the following manner:

- A continuous stimulation of 200 Hz for 1 s was applied and the twitch response recorded at various times during the post-tetanic period of about 10 min.
- Continuous stimulations during 30 s at 50, 100, 200 or 300 Hz were applied in that order after which twitch responses were recorded for about 30 min. Measurements were made of tetanic tension, twitch tension, twitch contraction time (cr) and half-relaxation time (hr) (Hansson and Lennernäs 1977, Lennernäs and Hansson 1978). EMG as recorded in five contractions using five thin platinum foil electrodes using on each side of the muscle.
- Test stimuli of 200 Hz for 0.5 s were used to examine the effects on the tetanic response of fatigue resulting from a stimulation rate of 200 Hz for 30 s.

Results

PTP of the twitch response Tetanic stimulation of a muscle over its nerve induces both fatigue and potentiation of the tension response. Potentiation can be of two types. Stimulations for several seconds at high rates are known to produce post-tetanic repetitive twitch activity in the nerve fibres, i.e. single pulse stimulation might lead to repetitive muscle contractions. This is often seen in slow but seldom in fast muscles of the cat hindlimb, at least in animals anaesthetized with barbiturates (Standæert 1964, Olsson and Swett 1971). Shorter stimulations at lower rates give rise to post-tetanic potentiation (PTP) of the excitation-contraction mechanism, seen most clearly in the twitch response of fast limb muscles (Brown and Euler 1938, Standæert 1964, Butler and Lewis 1965, Nyström 1968, Olsson and Swett 1971, Hansson 1974).

In order to check PTP of the inferior oblique twitch response, a short (1 s) burst at 200 Hz

The postnatal development of the inferior oblique muscle of the cat

II Effects of repetitive stimulation on isometric tension responses

By

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Abstract

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The changes with postnatal age in post-tetanic potentiation (PTP) and fatigue of the inferior oblique muscle have been studied in the cat. PTP of the twitch amplitude increased steadily with age up to 28 weeks of birth. Twitch contraction time (cr) and half relaxation time (hrr) was not significantly changed. The facilitation of the tetanic response also became more prominent with age. The young muscles (10 weeks below) were slightly more susceptible to fatigue than older muscles, but recovery was rapid in muscles of all ages. Intensive stimulation induced post-tetanic depression of twitch responses in muscles older than 1 week, but none in the muscles of new-born cats. Cr and hrr were greatly prolonged in muscles older than 1 week of age. In these muscles, but never in the very youngest, repetitive firing could be observed in response to post-tetanic single nerve stimulation. The results are compared with those obtained in experiments on hind-limb muscles by other workers. They further support the idea raised in a previous paper that slow muscle fibres develop quicker and reach maturity earlier than fast fibres.

Key words: Postnatal development, cat extraocular muscle, fast and slow fibres, PTP, fatigue

In a previous paper twitch and tetanic response properties of eye muscle were examined during postnatal development (Lennerstrand and Hanson 1978). Important differences were noted in the development of slow and fast fibre properties, and it was suggested the slow fibres reached maturity earlier than the fast fibres. This paper concerns the relations with age in the effects of repetitive stimulation on isometric contraction, i.e. fatigue and post tetanic potentiation (PTP).

In the adult cat most motor units in eye muscles are fatigue resistant. Only a few of the fastest units fatigue quickly (Lennerstrand 1974). However, it is not known how fatigue properties change in eye muscles during development.

Both fast and slow hind limb muscles, which have been better studied in this respect, showed fairly high resistance to fatigue from birth to about three weeks of age. After that period the fatigue resistance of slow twitch muscle increased slightly while the



1. Post-tetanic effects on the twitch size, expressed in percent of the pre-tetanic, recorded at different times after ceasing stimulus of 200 Hz for 1 s. Values are plotted for the age groups of 6 w, cats and adult. Standard deviation: 0 ± 11 , 6 ± 1 , 37 ± 6 , 29 ± 12 .

size and recovery of the tetanic response Fatigue was induced by continuous stimulus for 30 s at 50, 100, 200 or 300 Hz. Figure 4 A displays the response to a 100 Hz stimulus of three different muscles: one from a newly born kitten and the others from a six week kitten and an adult cat.

In the older muscles the tension output increased steadily during the initial part of the stimulation, the so called stair-case phenomenon. The stair-case effect was seen at 50 and 100 Hz but not at 200 and 300 Hz. For the same rate of stimulation it was of longer duration in older animals than in young ones. This is seen in Fig. 5 which displays the relative tension percent of the initial tension, recorded after 1, 3, 10 and 30 s of stimulation at various times, in kittens of three different age groups and in the adult cats. An adult pattern in stair effects was attained at 20 weeks of age.

The tension remaining after 30 s of continuous stimulation, expressed in percent of the tension at stimulus initiation, was used as a measure of fatigue resistance. With stimulations at 50 Hz the stair-case effects dominated the response and no fatigue was recorded (Fig. 5). At 100 Hz the fatigue was much more pronounced in the young (0- weeks) than in the

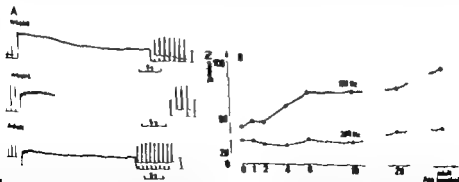


Fig. 4 A. The tetanic responses to 30 s, 100 Hz stimulation, preceded and followed by twitch responses, muscles from newborn (0 w), six weeks old and an adult cat. Tetanic tension recorded on bottom trace, together with twitches at low gain. Top trace shows pre- and post-tetanic twitches at high gain. Fatigue bar (at extreme right) represents for 0 w: 5 g, 6 w: 10 g and adult: 20 g. B. Fatigue resistance to 100 Hz stimulation for 30 s at 100 and 200 Hz in muscles of different ages. Fatigue resistance expressed as the tetanic tension remaining after 30 s continuous stimulation, in percent of the tetanic tension at stimulus initiation. Average values plotted; 5 Hz for 100 Hz stimulation: $\pm 10.1\%$ and 200 Hz: $\pm 5.4\%$.

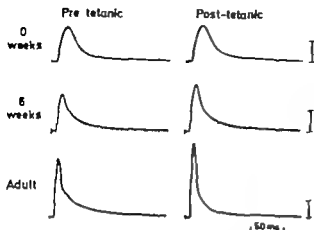


Fig. 1 Twitch contractions of muscles from a new-born (0 w), a 6 weeks old and a fully grown cat, recorded pre-tetanically and 15 s after a potentiating tetanic 200 Hz stimulus of 1 sec duration. The tension scales (on the right) represent 0.2 g for the 0 and 6 kittens, and 1 g for the adult cat.

was applied to the nerve. Fig. 1 shows the twitch response before and 15 s after the tetanic stimulation of a 2 day old, a 6 week old and an adult muscle. PTP occurred in all muscles but was much less pronounced in the newborn kitten. As seen in Fig. 2 A the immediate (15 s post-stimulatory) effect on the twitch amplitude became increasingly larger with age. The difference between age groups below 4 weeks and above 20 weeks is significant at the 1% level. The contraction time (*cr*) of the twitch response was only slightly affected. The half-relaxation time (*hrt*) was somewhat shorter than pre-tetanically at ages above 1 week (Fig. 2 B) but these variations were not statistically significant. The post-tetanic changes in the twitch amplitude during a post-stimulatory period of 10 min are shown in Fig. 3. In the two youngest age groups the twitch amplitude was slightly reduced below the pre-tetanic value, after an initial potentiation lasting for about 1 min. The twitch did not fully recover in these muscles within the observation time (10–12 min). In the older animals (6 weeks and older) the twitch amplitude was potentiated for 2–5 min without post-stimulatory depression (Fig. 3).

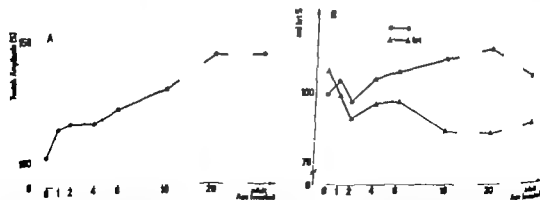


Fig. 2. Post-tetanic effects on the twitch response, induced by 200 Hz stimulation for 1 s, plotted against age. The amplitude (in A), *cr* and *hrt* (in B) have been measured in twitches recorded 15 s post-tetanically and have been expressed in percent of the corresponding parameter of the pre-tetanic twitch. Average values for each age group are given. The standard deviation was about the same for all groups and the overall values calculated in an analysis of variance amounted to $\pm 9.3\%$ for twitch amplitude, $\pm 8.1\%$ for *cr* and $\pm 8.3\%$ for *hrt*.

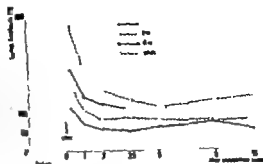


Fig. 3. Post-tetanic effects on the twitch tension, expressed as percent of the pre-tetanic values, recorded at different times after voluntary stimulation of 200 Hz for 1 s. Age values are plotted for the age groups and 6 weeks and adult. Standard deviations: $0w \pm 5.1$, $3w \pm 3.7$, $6w \pm 2.9$, adult: ± 1.2 .

Fatigue and recovery of the tetanic response Fatigue was induced by continuous stimulation for 30 s at 50, 100, 200 or 300 Hz. Figure 4 A displays the response to a 100 Hz stimulation of three different muscles: one from a newly born kitten and the others from a six week kitten and an adult cat.

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The tension remaining after 30 s of continuous stimulation, expressed in percent of the tension at stimulus initiation, was used as a measure of fatigue resistance. With stimulations at 50 Hz the stair-case effects dominated the response and no fatigue was recorded (Fig. 5). At 100 Hz the fatigue was much more pronounced in the young (0-6 weeks) than in the

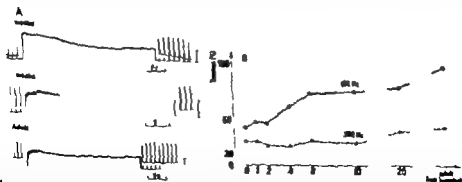


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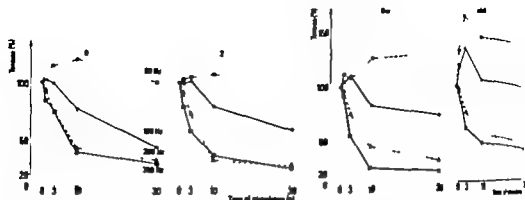


Fig. 5. Graphical display of tension development during continuous stimulations for 30 s at 50 (\circ), 200 (Δ) and 300 Hz (\square). Tension measured after 1, 5, 10 and 30 s of stimulation and percent of tension at stimulus initiation. Average values plotted for the age groups of 0, 2 and 6 weeks and the adult animals.

adult cat (Fig. 4 B) but even here stair-case effects influenced the results. At rates of stimulation of 200 and 300 Hz, when no stair-case effect was seen, muscles in all animals below 10 weeks of age exhibited about the same amount of fatigue (Fig. 4 B). The 20 week old adult muscles had a significantly higher fatigue resistance than the others ($0.01 < p < 0.05$).

The recovery of the tetanus response from fatigue was also followed. After a continuous 200 Hz stimulation for 30 s the response to a tetanic burst of 200 Hz for 1 s was lower than prior to the fatiguing stimulation. This is shown in Fig. 6 for a muscle of an adult cat. Within 1 min the magnitude of the tetanic response had returned to the value it had before the continuous stimulus was introduced. Figure 6 B displays graphically the recovery of tetanic tension in muscles of different ages. Muscle tension was recorded at 2 s, 15 s and 1 min after the 30 s stimulation. The tension drop during the first seconds was most marked in the young animals but at all ages, except in the groups of 6 week and 10 week old animals, the recovery was complete in less than one minute. At 6 weeks the fatigue effect lasted for 5 min and at 10 weeks for 8–10 min. This difference was significant at the 5% level.

Twitch responses after long stimulation. The effects of the long stimulations on the twitch response consisted of a mixture of potentiation and depression, probably of different time courses. This is exemplified in Fig. 7 A, where the magnitude of the twitch amplitude at different times after the conditioning stimulus (200 Hz for 30 s) has been plotted for muscles of new-born, 1 week old, 6 week old and adult cat. For all muscles except the very youngest (0 week), the twitch amplitude after an initial increase dropped markedly below the pre-stimulatory value. In the youngest muscles only potentiation and no depression of the twitch was seen (Fig. 7 A). It lasted for 5–8 min irrespective of the frequency of potentiation stimulus. In the muscles from animals older than 1 week, a depression of the twitch amplitude appeared within the first minute post tetanically and the tension returned slowly to the level before stimulation over the following 10–20 min. The time for full recovery of the twitch amplitude after a 200 Hz tetanization was about 12 min in 4 week old kittens and 15–20 min in 6, 10 and 20 week old kittens and the adult cat.

A post tetanic increase was seen in the *ct* and *hrt* values of all muscles as shown in Fig. 7 B and C for stimulations at 200 Hz for 30 s. *ct* and *hrt* returned to pre-stimulatory values

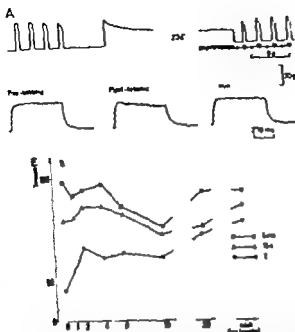
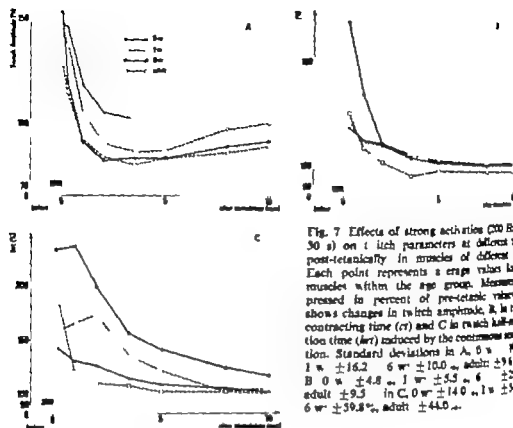


Fig. 6. Recovery from fatigue of the tetanic responses to 200 Hz for 0.5 s. The test stimuli were tetanic contractions at 200 Hz for 0.5 s and the fatigue stimulation 200 Hz for 30 s, exemplified for an adult preparation. A: traces show tetanic responses to test stimulations pre-tetanically and at 15 s and 1 min post-tetanically as an expanded time scale. In B is shown the recovery of the tetanic response in different age groups. The test tetanus produced by the test stimulations was measured 2 s, 15 s and 1 min after the fatigue stimulation and expressed in percent of the pre-tetanic value. Recovery back to the 100%-level occurred rapidly in muscles of all ages, although the initial depression of the response was higher in young than in old muscle. Data values given in B, standard deviations for error: at 2 s $\pm 8.8\%$, 15 s $\pm 3.2\%$ and 1 min $\pm 3.7\%$.

within 10 min in the newly born and the one week old kitten, as well as in the adult animal. In animals 6 weeks of age the increase was larger than in the other age groups ($0.01 < p < 0.05$) and lasted longer (see Fig. 7 C). Similar patterns were observed for the 10 and 20 week old kitten.

In all age groups the largest twitch amplitude was obtained within 5 s from completion of continuous stimulation. The twitch response recorded at 15 s was analyzed with respect to age variations in amplitude, cr and hcr (Table I). The most prominent features were the large increases in cr and hcr in muscles above 6 weeks of age for conditioning stimulations at 200 and 300 Hz. These effects were less pronounced for 50 Hz stimulations but became clearly noticeable at 100 Hz as seen in Table I.

Double peaks were sometimes seen in the twitch curve during the first 2 min of the post-tetanic period (Fig. 8 A). Such twitch responses were most common in the 6-10 week old muscles. They did occur also in the muscles of the adult cat, but they were never encountered in the newborn animals. As shown by the EMG-recording in Fig. 8 B the second peak must have been caused by repetitive firing of the muscle in response to single shock stimulation of the nerve.



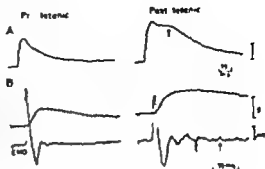
Discussion

Fatigue properties In the adult cat eye muscles are known to be highly fatigue resistant (Lennerstrand 1974, 1975). Eye muscles in the kitten showed a slightly lower endurance than adult muscle to the most intensive activations. The fatigue resistance remained at 6

TABLE 1. Twitch parameters measured 15 s after repetitive stimulation for 30 s at different rates, in percent (\pm S.D.) of pre-tetanic values.

Age (weeks)		1	2	4		10	20	Adult	
50 Hz	n	6	6	6	8	7	7	4	3
	ampl	149 (± 35)	119 (± 5)	127 (± 19)	124 (± 8)	113 (± 13)	134 (± 10)	159 (± 6)	141 (± 4)
	cr	1.3 (± 11)	118 (± 8)	116 (± 8)	126 (± 7)	124 (± 13)	129 (± 8)	133 (± 13)	114 (± 1)
	hcr	133 (± 15)	137 (± 21)	133 (± 23)	131 (± 13)	146 (± 41)	107 (± 14)	103 (± 70)	112 (± 1)
100 Hz	n	6	6	6	9	7	6	4	4
	ampl	131 (± 16)	121 (± 22)	127 (± 1)	117 (± 19)	112 (± 11)	126 (± 19)	134 (± 6)	121 (± 1)
	cr	117 (± 6)	121 (± 13)	134 (± 17)	146 (± 30)	167 (± 45)	149 (± 14)	135 (± 14)	139 (± 1)
	hcr	137 (± 5)	172 (± 69)	151 (± 35)	154 (± 31)	194 (± 29)	178 (± 30)	168 (± 47)	141 (± 1)
200 Hz	n	6	7	8	9	7	6	4	3
	ampl	146 (± 19)	138 (± 3)	135 (± 22)	130 (± 31)	130 (± 16)	113 (± 3)	144 (± 13)	120 (± 1)
	cr	118 (± 5)	121 (± 7)	121 (± 10)	145 (± 35)	169 (± 22)	153 (± 19)	135 (± 13)	123 (± 1)
	hcr	141 (± 33)	158 (± 66)	143 (± 23)	154 (± 79)	233 (± 68)	222 (± 58)	184 (± 63)	184 (± 1)
300 Hz	n	4	5	6	8	7	7	4	3
	ampl	153 (± 21)	150 (± 19)	130 (± 2)	140 (± 21)	140 (± 14)	135 (± 11)	157 (± 7)	176 (± 1)
	cr	117 (± 5)	120 (± 8)	121 (± 13)	144 (± 23)	168 (± 21)	144 (± 10)	149 (± 9)	114 (± 1)
	hcr	142 (± 13)	153 (± 67)	141 (± 37)	164 (± 19)	223 (± 37)	216 (± 75)	207 (± 55)	183 (± 1)

Fig. 1. A. Post-tetanic twitch response in adult cat recorded 15 min after potentiation by 30 s 200 Hz stimulation showing the peaks (arrows) seen in the pre-tetanic responses. B. Twitch response EMG recorded pre- and post-tetanically in adult cat. Repetitive firing is seen post-tetanically (arrows) after a 30 s stimulation 50 Hz.



level from birth up to 20 weeks of age when adult values were reached. Strong muscle contractions lasting for minutes might reduce the blood flow to the muscle and render it ischemic, thereby influencing the normal fatigue properties (Hammarberg and Kellerth 1975a). It cannot be excluded that such effects might have been introduced in the youngest litters by the 30 s stimulations, but in the older muscles and in the adult cat the rates and periods of constant muscle fibre activation do probably not exceed those that occur under natural conditions (cf. Yamanaka and Bach-y-Rita 1968, Lennestrand 1974). The recovery from fatigue after the long-lasting stimulation was rapid at all ages, except at 6 weeks and 10 weeks of age.

With the lower stimulus rates used (50 and 100 Hz) potentiation of the tetanic response occurred. This gave rise to a stair-case phenomenon that reduced or even concealed the true effects of the long-lasting stimulation. The stair-case effect was more prominent in the 6 week or older animals than in the younger kittens.

Adult cat eye muscle has a mixed population of slow and fast motor units. All of the slow units and the majority of the fast units were highly fatigue resistant (Lennestrand 1974). If the total tension response of eye muscles at all ages reflected the combined activity of individual motor units as directly as in hindlimb muscle (Hammarberg and Kellerth 1975b), the proportion of fatigue resistant to fatigue sensitive units would seem to rise slightly with age, at least during the first 20 weeks of postnatal development.

PTP and development of fast and slow fibres. In a previous paper (Lennestrand and Jansson 1977) evidence was presented in support of the idea that slow fibres in cat eye muscles are more or less fully developed at 6-10 weeks of age, while the fast fibres did probably not reach maturity until the cat was 1/2-1 year old. PTP of the twitch response might provide another means by which differences in the rate of maturation between fast and slow fibres can be studied.

Eye muscles contain slow fibres that are multiply innervated and show several end-plate regions on the same fibre (Bach-y-Rita 1971, 1975, Lennestrand 1974, Alvarado and van Horn 1975), while all slow fibres of hindlimb muscles are focally innervated with only one end-plate region per fibre. Unfortunately there are no studies available on PTP in slow multiply innervated fibre systems, neither in the mature nor in developing muscles. Provided PTP effects in the multiply innervated fibres of eye muscles are similar to those of slow fibres in hindlimb muscles, the PTP results in developing eye muscles could be interpreted as

follows (i) PTP increased in eye muscles up to 20 weeks of age, indicating that basic properties were developing over this period (Buller and Lewis 1965 Nyström 1968). The change occurred in parallel with the increase in speed of twitch contraction, used in a previous paper (Lennerstrand and Hanson 1978) as a sign of fast fibre development, (ii) strong and long lasting activations were applied the shape of the twitch response distorted due to post-tetanic repetitive firing in the muscle. These effects were not seen in the adult animal but became more and more marked in older muscles and reached their maximum 6–10 weeks of age. Repetitive firing has been observed only in slow muscle (Standen 1968 Nyström 1968 Olson and Swett 1971 Stephens and Stuart 1975). The findings in eye muscle would indicate that slow fibres were more or less fully developed at age 10 weeks, and the later changes in muscle characteristics would be caused by the further development of fast fibres.

The early development of the slow fibre system in eye muscles may be connected with demands for slow movements and sustained motor activity in fixation and binocular vision. The visual functions of the cat reach maturity around the age of 10 weeks (Blakemore and van Sluyters 1975) and at this age the slow fibre component of extraocular muscle would seem fully developed.

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Binding of thiamine to nicotinic acetylcholine receptor in *Torpedo marmorata* and the frog end plate

By

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Received 29 November 1977

Abstract

WALDENLIND L., L. ELFMAN and B. RYDQVIST. *Binding of thiamine to nicotinic acetylcholine receptor in *Torpedo marmorata* and the frog end plate*. Acta physiol. scand. 1978. 103: 154-159.

Thiamine was found to bind to the isolated nicotinic receptor (nAChR) from *Torpedo marmorata* ($K_D = 3-5 \times 10^{-4}$ M). The binding was reversible and inhibited by α -neurotoxin from *Naja naja siamensis*. Acetylcholine binding was not inhibited by thiamine. When thiamine (5×10^{-4} M) was applied to sartorius muscles a decrease of miniature end plate potential amplitudes was seen. This effect was reversible after the perfusion fluid of the muscle was changed to normal Ringer solution. Two biological and electrophysiological studies indicated a binding of thiamine to the nAChR.

Abbreviations: ACh = acetylcholine, MEPP = miniature end plate potential, nAChR = nicotinic acetylcholine receptor and PTP = post-tetanic potentiation.

It has been shown that post tetanic potentiation (PTP) is abolished in thiamine deficit induced by pyriethamine (Waldenlind 1977). The PTP was then recovered by thiamine injections. The importance of thiamine for PTP was not correlated to the cocarboxylase reactions of thiamine-diphosphate. Besides it was shown that di-tubocurarine in doses (4 μ M) which left the ordinary supramaximal muscle twitches unaffected, blocked the PTP. Therefore it was assumed that thiamine was necessary for curare sensitive receptors mediating PTP. This assumption led us to study the binding of thiamine to the purified nAChR from *Torpedo marmorata* and the electrophysiological effects of thiamine on miniature end plate potentials (MEPP's) registered from the muscle of the frog *Rana pipiens*.

Methods

Binding of thiamine to nAChR

The nAChR from *Torpedo marmorata* electric organ was purified by biospecific chromatography: acetylated *Naja naja siamensis* toxin bound to Sepharose 4B (Heilbronn and Mattsson 1974). Carboxyl elution gave a protein peak, which was further purified on a DEAE-ion exchanger in order to get rid of carbachol and traces of acetylcholine esterase.

drug of (^{35}S) thiamine hydrochloride (10^{-4} – 10^{-6} M) to the nAChR tested earlier described, both the Sartorius membrane filter method (Hedbrook and Mattsson 1974) and equilibrium dialysis (Korn et al. 1974). The nAChR (1 mg/ml) was incubated with neurotoxin α -neurotoxin, specific agent for nAChR, for 15 min at room temperature before the binding of thiamine as standard. One experiment the receptor was incubated with oxythiamine and pyrimethamine (up to 10^{-3} M) prior to the binding studies. Oxythiamine inhibits carbonylase function of thiamine-diphosphate but never in neurological symptoms. While pyrimethamine induces neurological symptoms resembling of thiamine deficiency without any major direct effect on carbonylase functions of thiamine-diphosphate (Stern 1967).

study whether thiamine could inhibit the binding of ACh, the receptor protein was first incubated with 1 M saline for 60 min at room temperature in order to block traces of acetylcholine esterase. Thiamine (10^{-3} M) was then added and the incubation continued for another 30 min. $(^{125}\text{I})\text{ACh}$ (10^{-6} M) in assay containing buffer was then added and equilibrium dialysis was performed. Assay was determined according to Lowry et al. (1951).

Electrophysiological recordings

Recording of MEPP is performed on sartorius muscle fibres from small *Rana pipiens*. The muscle dissected free and placed in perspex chamber (volume 5 ml) with its deep surface exposed. The Ringer bath contained 115 mM NaCl, 5 mM KCl, 3 mM CaCl_2 , 2.5 mM Na_2HPO_4 and 0.85 mM NaH_2PO_4 , the pH is 7.0–7.2 (Adrian 1954). The solution in the chamber is changed carefully with continuous rising from waste fibres. The location of the end-plate region is determined by visual inspection using binocular microscope. 100 \times dark field illumination. Intracellular potential changes were elicited using glass microelectrodes filled with 3 M KCl and with tip resistance of 8–10 M Ω . The microelectrode was connected to high input impedance ($>10^9$ Ω) amplifier. The output from this amplifier is fed to an oscilloscope (Tektronix 5103 N), digital oscilloscope (to record resting membrane potential) and pen 238 amplifier (AC-coupled, with low cut-off frequency (<3 dB point) of 0.3 Hz) the output lock was fed to tape-recorder (Hewlett-Packard 3940). The speed of the tape-recorder is set to 1 inch lock equivalent to bandwidth of 0–1250 Hz. The MEPP then stored on tape could later displayed on an oscilloscope and recorded on photographic film.

Materials

ACh (250 $\mu\text{Ci}/\text{mmol}$) and (^{35}S) thiamine (150 $\mu\text{Ci}/\text{mmol}$) are purchased from the Radiochemicals, Amersham, U.K. Oxythiamine and pyrimethamine were obtained from Sigma Chemical Co. Other chemicals are from usual commercial sources.

Results

Binding of thiamine to nAChR

re purified nAChR protein from *Torpedo marmorata* which has a specific affinity for allosteric ligands (Chang and Lee 1966, Hedbrook and Mattsson 1974, Neuner et al. 1974) is also able to bind thiamine *in vitro* (Fig. 1 and 2). The K_D -value for the binding of thiamine was shown to be $3.05 \pm 0.87 \times 10^{-6}$ M when measured by the Sartorius membrane filter technique (Fig. 1) and $5.10 \pm 2.63 \times 10^{-6}$ M when measured by equilibrium dialysis (Fig. 2). The maximum binding capacity was 0.84 ± 0.19 nmol/mg receptor protein when Sartorius (filter technique) was used (Fig. 1) and 2.57 ± 0.79 nmol/mg protein when measured by equilibrium dialysis (Fig. 2). All values are mean \pm S.D.

The binding of thiamine to the nAChR was inhibited by the nAChR antagonist α -neurotoxin from *Naja naja siamensis* at a concentration of 10^{-6} M. This was proven by both techniques. The binding of ACh (10^{-6} M) was not inhibited by thiamine (10^{-4} – 10^{-6} M), nor did the presence of thiamine (10^{-6} M) change the K_D -value (2.3×10^{-6} M) for the binding of ACh (checked by equilibrium dialysis).

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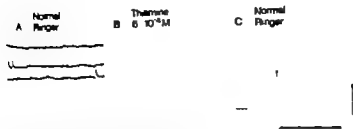


Fig. 3. Effect of thiamine on MEPP's. A. Before application of thiamine. B. Two min after application of 5×10^{-6} M. C. After return to Normal Ringer. Resting membrane potential: (A) -93 mV (B) -90 mV (C) -94 mV. Time bar: 0.5 s, Vertical bar: 2 mV.

the resting membrane potential was not affected in thiamine solutions, the values ranging from about -90 mV to -95 mV. After the final measurements in normal saline, the microelectrode was withdrawn from the fibre and the resting membrane potential was found to be -94 mV.

Discussion

Isolated nAChR from *Torpedo marmorata* binds thiamine at a concentration of 10^{-6} M. Binding of thiamine is prevented by α -neurotoxin from *Agelena saeva stenocera*, which is a

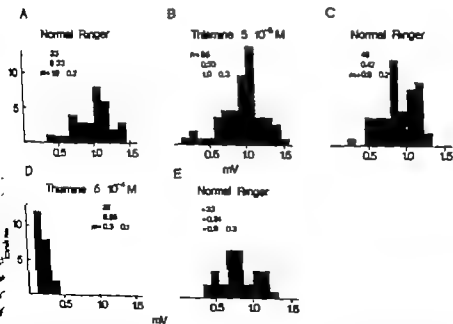


Fig. 4. Histograms from experiments as illustrated in Fig. 3 showing distribution of amplitudes of MEPP's in muscle fibre treated with two concentrations of Thiamine (5×10^{-6} M and 3×10^{-6} M). n = number of fibres, / mean MEPP frequency (s^{-1}), \pm standard deviation of MEPP amplitude (mV). Resting membrane potential: (A) -92 mV (B) -90 mV (C) -93 mV (D) -95 mV (E) -94 mV.

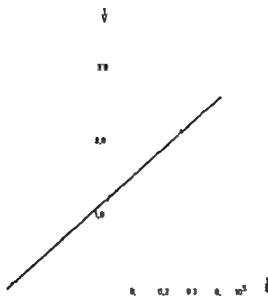


Fig. 1

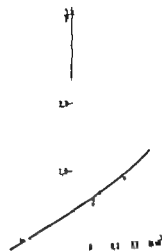


Fig. 2

Fig. 1. Lineweaver Burk plot showing binding of thiamine to nAChR using the Sartorius microtitre method. The K_D -value calculated from the curve is $3.05 \pm 0.87 \times 10^{-6}$ M and the maximal binding capacity 0.84 ± 0.10 nmol of thiamine per mg of protein.

Fig. 2. Lineweaver Burk plot showing binding of thiamine to nAChR using equilibrium dialysis. The K_D value calculated from the curve is $5.10 \pm 2.63 \times 10^{-6}$ M and the maximal binding capacity $2.57 \pm 0.9 \mu$ mol of thiamine per mg protein.

Oxythiamine and pyrithiamine (two thiamine antagonists) were tested in order to determine whether thiamine binding to nAChR could be prevented. No inhibition could be seen at concentrations up to 10^{-4} M.

Electrophysiological recordings

Thiamine (10^{-6} – 5×10^{-4} M) was applied to frog sartorius muscles for different periods of time (2–10 min mainly depending on the concentration used). Up to a concentration of 10^{-6} M no changes in mean amplitude of the MEPP's could be seen. At higher concentrations (1.5×10^{-4} M) a decrease in MEPP amplitude was observed (Fig. 3). Amplitude histograms for spontaneous MEPP's in the same fibre as in Fig. 3 are shown in Fig. 4 at two concentrations of thiamine (5×10^{-6} and 5×10^{-4} M). At 5×10^{-6} M the mean amplitude was unchanged (at 95% significance level) compared with the mean amplitude in control measurements both before and after application of thiamine (Fig. 4 A, B and C). After application of 5×10^{-4} M thiamine, the mean MEPP amplitude was drastically reduced from 0.9 mV (Fig. 4 C) to 0.3 mV (Fig. 4 D). At this concentration, reduction occurred within about 1 min, the total time of application being 3 min. After 5 min in normal Ringer solution the MEPP amplitude had returned to the same value (0.9 mV) (Fig. 4 E) as before application of thiamine (no difference at a 95% significant level). The MEPP frequency seems to be somewhat increased at both concentrations of thiamine compared with the frequency in Ringer solution (Fig. 4).

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specific antagonist of the nAChR (Chang and Lee 1966). The binding of thiamine to isolated nAChR occurs in a similar dose range to that which inhibits nicotine-induced contractions of the guinea-pig ileum (Waldenlind 1977). The K_D -value for thiamine in the present study is based on *in vitro* experiments. The binding constants need not be the same *in vivo*. It is known that the binding of e.g. ACh to the isolated nAChR occurs at a K_D which is higher than that which is active on isolated organ preparations. Extraneous factors such as temperature, pH, intact membrane structure and possible co-factors present, etc., are defined in the non-injured tissue, may change the binding constant. Therefore the K_D value gives no information concerning the concentration of thiamine which could be active *in vivo*. Thiamine decreased the mean MEPP amplitude in the frog end plates without affecting the membrane potential when added in a concentration of $5 \cdot 10^{-4}$ M (Fig. 3). At this concentration about 85% of all thiamine binding sites of the nAChR from *Torpedo marmorata* are occupied. This electrophysiological effect may therefore be explained by being due to binding of thiamine to the nAChR of the end plates. The bulk concentration of thiamine ($5 \cdot 10^{-4}$ M) necessary to block the MEPP's is probably higher than the concentration at the end plate region due to the high polarity of the thiamine molecule which makes it difficult to penetrate tissue barriers.

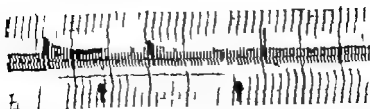
In the purified nAChR the high affinity site ($K_D = 3 \cdot 10^{-8}$ M) is changed to a low-affinity site ($K_D = 2 \cdot 10^{-6}$ M) for ACh. Thiamine could not inhibit the low-affinity binding of ACh to purified nAChR but in electrophysiological studies where only the high-affinity site is present, the results indicate an effect on either the acetylcholine binding or the ionophore activity of the nAChR.

The electrophysiological studies do not reveal any certain physiological role for thiamine at the post-synaptic nAChR. However, it seems clear that thiamine binds to nAChR and this has been shown by binding studies on isolated receptors and by electrophysiological recordings. PTP is mediated by the activation of presynaptic curare-sensitive receptors and thiamine is necessary for the function of these receptors (Waldenlind 1977, Waldenlind To be published). It is thus possible that the physiological role of thiamine may be in the mediation of PTP through activation of curare-sensitive receptors.

We wish to thank Professor E. Heilbronn for helpful comments on the manuscript.

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1 Effect of hexamethonium on PTP. The first arrow indicates the infusion of hexamethonium (7.0 mg , part of the PTP is still left. No effect on the single twitches is seen. The post PTP is, however, significantly smaller. The second two arrows indicate the infusion of an additional dose of hexamethonium (10.5 mg/kg). PTP now totally abolished and the single twitches are still the same as in the beginning. The contraction force is equivalent to 35 g .

Methods

In Sprague-Dawley rats ($250\text{--}300 \text{ g}$) were anesthetized with urethane sodium (30 mg/kg). The trachea intubated with plastic tube just below the larynx. The external jugular vein is cannulated to allow anesthesia. The portal blood circulation of the muscular vesicle was intact. The sympathetic nerve was opened and cut off proximally. Sympathetic stimulation (1.5 times that required for maximal stimulation) of the sympathetic nerve was then carried out both directly and by field stimulation (0.2 Hz , 0.2 sec) by Grass SD 9 stimulator using platinum electrodes. The electric stimulus of the muscular vesicle were carried by current through the metal electrode bone. The thread is connected to Grass force-displacement meter (FT 10) and Grass polygraph (model 7 B). The contraction force registered is therefore proportional to the tension in the muscular vesicle. The initial tension was equivalent to 150 g . The contractile force is given in the individual figures. The PTP is provoked by brief tetanic stimulation at $112 \text{ for } 5 \text{ s}$, for the contracted muscle responded with individual twitches at frequencies at 20 Hz (typical for fast and the first PTP sometimes occurred). However it is an accepted way of describing the phenomenon itself. All preparations are dissolved in 150 mM NaCl (0.3 ml) and exposed.

Materials

Hexamethonium chloride and hexamethonium bromide were obtained from Sigma Chemical Co. (Sweden) and sodium pentobarbital from ACD. Prostigmin and dantrolene from Hoffmann-La Roche, neostigmine from Vitarum, Sweden and haloperidol from LEO, Sweden.

Results

Hexamethonium inhibited PTP dose-dependently. The ordinary twitches were unaffected (Fig. 1). Prostigmin ($1.5 \text{ } \mu\text{g/kg}$) did not reverse the effect of hexamethonium, on the contrary a potentiation of the hexamethonium effect was noted. PTP was inhibited by doses of hexamethonium (ED_{50} $1.75\text{--}2.0 \text{ mg/kg}$, $n=4$) slightly higher than those inhibiting the action of nicotine on the isolated guinea-pig ileum (Waldenlund 1977).

Succinylcholine blocked PTP dose-dependently in doses (ED_{50} $5\text{--}7 \text{ } \mu\text{g/kg}$, $n=4$; ED_{100} $9\text{--}13 \text{ } \mu\text{g/kg}$, $n=4$) which did not affect the ordinary muscle twitches (Fig. 2). At higher doses the ordinary muscle twitches were reduced (ED_{50} $40\text{--}50 \text{ } \mu\text{g/kg}$, $n=4$). The effect of succinylcholine on the PTP could not be reversed by prostigmin. The ordinary muscle twitches increased after prostigmin administration at a dose when the PTP was totally inhibited by succinylcholine showing that the end plate receptors were unaffected (Fig. 2).

Pharmacological properties of curare-sensitive receptors mediating post tetanic potentiation in rat masseter muscle

By

LENNART WALDENLIND

Received 29 November 1977

Abstract

WALDENLIND L. *Pharmacological properties of curare-sensitive receptors mediating post tetanic potentiation in rat masseter muscle* Acta physiol. scand. 1978. 103 160-164

Post-tetanic potentiation (PTP) of single muscle twitches after "tetanic" stimulation at 1 to 20 Hz in rat masseter muscle has been studied. PTP was inhibited by d-tubocurarine (2-6 $\mu\text{g/kg}$), hexamethonium (1.75-2.5 mg/kg), succinylcholine (3-7 $\mu\text{g/kg}$), prostigmin (1.0-1.3 $\mu\text{g/kg}$), lidocaine (3-4 mg/kg) and meprobamate (215-440 $\mu\text{g/kg}$). The doses did not affect the control twitches. Prostigmin could not reverse the inhibitory effect on the PTP caused by these drugs. Atropine was without any effect on the PTP. The nicotinic receptors mediating PTP differ from both end-plate and ganglionic receptors since drugs blocking both types of receptors are effective. The sensitivity to the blocking agents is higher or the same (in the case of hexamethonium) as for the two other types of nicotinic receptors. Thus the PTP is dependent on an action of a "nicotinic" receptor active during physiological conditions, suggesting the presence of an additional mechanism for neuromuscular transmission in rat twitch fibres.

Tetanic stimulation of fast skeletal muscle causes a transitory increase in the peak force of the isometric twitch response (post tetanic potentiation, PTP), whereas a decrease is noted in slow mammalian muscles (Brown and Euler 1938, Bowman 1962, Standen 1966 and Close and Hoh 1968). PTP is absent in muscles perfused with saline solutions (Brown and Euler 1938) suggesting the importance of a humoral factor. It has been shown that PTP is abolished in thiamine-deficiency induced by pyridoxamine, or by d-tubocurarine in doses lower than those affecting the normal single twitches (Waldenlind In Press). Since thiamine has been shown to bind to nicotinic receptors (Waldenlind *et al.* In Press) and since the role of thiamine for the development of PTP was found to be unrelated to the decarboxylase functions of thiamine-diphosphate, it was thought that thiamine is important for the function of curare-sensitive receptors necessary for the occurrence of PTP. In the present article the pharmacological properties of curare-sensitive receptors mediating PTP are studied.

4 The effect of atropine on Atropine (75 μ g/kg) has been added after the first tetanus and that on either the single twitches or the PTP can be seen. tetanic contraction force is related to 63 g.



Discussion

results show that PTP is inhibited by nicotinic receptor blocking agents in doses lower than those affecting the ordinary muscle twitches. Inhibition of PTP occurred both with tubocurarine and hexamethonium indicating that the "nicotinic" receptors mediating are undifferentiated and thus constitute a third class of nicotinic receptors active under physiological conditions. In Table I it is clearly seen that nicotinic receptors mediating PTP in fast mammalian twitch muscles are pharmacologically different from end plate receptors and nicotinic receptors mediating post-tetanic repetition in slow muscles, such as cat soleus muscle. This difference is also seen in Fig. 2 where PTP is totally inhibited, but still prostigmin could increase the ordinary twitches. The end plate receptors must therefore be unaffected since succinylcholine blocks ordinary muscle twitches irreversibly in the case of curare and lidocaine, the single twitches could always be increased even if PTP was fully inhibited. Since the increase in single twitches after prostigmin treatment is due to post-tetanic repetition, PTP and post-tetanic repetition seem to have different pharmacological characteristics.

It is probable that the action of lidocaine is on nicotinic receptors since a small dose of tubocurarine which has no effect itself on the PTP potentiates the action of lidocaine or curare. Presynaptic nicotinic receptors have been isolated which bind both local anesthetics and curare in low concentrations (Denburg and O'Brien 1973).

TABLE I The table summarizes the effects of drugs on end plate receptors, nicotinic receptors mediating PTP in fast mammalian muscles and nicotinic receptors mediating post-tetanic repetition. The effects of drugs on post-tetanic repetition are from Raker (1969).

	end plate receptors	PTP	post-tetanic repetition
tubocurarine	reversible inhibition	nonreversible inhibition	reversible inhibition
hexamethonium	0	nonreversible inhibition	0
succinylcholine	depolarizing nonreversible inhibition	nonreversible inhibition	stimulation
prostigmin	stimulation (by AChE-effect)	nonreversible inhibition	stimulation
atropine	0	0	0
lidocaine	inhibition	inhibition	inhibition
curare	0	0	depression
curare	0	nonreversible inhibition	?

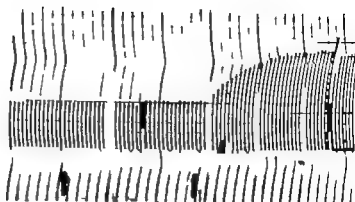


Fig. 2. Effect of succinylcholine and prostigmin on the PTP. The first arrow indicates the administration of succinylcholine ($10 \mu\text{g/kg}$). The PTP is almost completely abolished. The second bar shows the effect of prostigmin ($6 \mu\text{g/kg}$). The single twitches at low frequency stimulation increase but PTP is still abolished. The pretetanic contraction force is equivalent to 44 g .

Lidocaine also inhibited the PTP dose-dependently ($\text{ED}_{50} = 3\text{--}4 \text{ mg/kg}$, $n = 5$) as did ouabain ($\text{ED}_{50} = 215\text{--}240 \mu\text{g/kg}$, $n = 5$) (Fig. 3). Both lidocaine and ouabain completely inhibited PTP without any effect on the ordinary twitches. Pretreatment with a dose of d-tubocurarine which was about half of that having a minimal effect on the PTP gave a dose-dependent inhibition of the PTP by lidocaine ($50\text{--}100 \text{ mg/kg}$) in doses which otherwise were without effect. The inhibition of the PTP by lidocaine or ouabain was not reversed by prostigmin. The single twitches could always be increased by prostigmin even if the PTP was inhibited.

Prostigmin inhibited PTP dose-dependently ($\text{ED}_{50} = 1.0\text{--}1.5 \mu\text{g/kg}$, $n = 4$; $\text{ED}_{50} = 2.5 \mu\text{g/kg}$, $n = 4$). The ordinary muscle twitches began to increase at a dose of $3\text{--}4 \mu\text{g/kg}$.

Atropine was without any effect on the PTP in doses up to 50 mg/kg (Fig. 4). Haloperidol (10 mg/kg), mebumal (50 mg/kg) and diazepam (2 mg/kg) were without any effect on PTP.

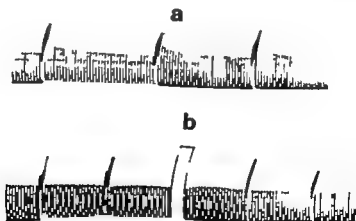


Fig. 3. Effect of ouabain on PTP. a) PTP after stimulation at 20 Hz for 5 s of untreated preparation. Tetanic stimulations at 30 Hz for 5 s and at 50 Hz for 20 s after infusion of $120 \mu\text{g/kg}$ ouabain. No PTP develops after stimulation at 20 Hz or 50 Hz (for 20 s). The pretetanic contraction force is equivalent to 41 g . The 50 Hz stimulation for 20 s reached the maximum level of the Grass. Therefore the maximum contraction force can not be calculated.

Rabbit lung plasma and erythrocyte volumes. Lung hematocrit in relation to total body hematocrit

By

P. AARSETH, B. A. WAALER and G. NICOLAYSEN

Received 5 December 1977

Abstract

AARSETH, P., B. A. WAALER and G. NICOLAYSEN. *Rabbit lung plasma and erythrocyte volumes. Lung hematocrit in relation to total body hematocrit*. Acta physiol. scand. 1978, 103, 165-172.

Total body hematocrit has been reported to be 83-90% of packed cell volume (PCV) in several species. We have found similar values in rabbits. An extra plasma volume must exist somewhere in the vascular system to explain this observation. We have looked for such an extra plasma volume in the pulmonary circulation. The dynamic hematocrit was estimated in isolated, perfused rabbit lungs from distribution volumes for plasma and erythrocyte tracers. Estimation was also obtained from indicator-dilution curves and bolus-injection of each tracer avoiding their recirculation. It was then possible to calculate mean transit times for the tracers from their dilution curves directly or applying monoexponential extrapolation on the first part of the downslope of the curves. The dynamic hematocrit of the lung vessels is about 1% of packed PCV and there is no difference between the results obtained by the different methods. We concluded that in the rabbit only a very small part of the extra plasma volume is located in lung vessels. The lung plasma volume is not overrepresented by the indicator-dilution technique.

The plasma volume in a mammal is larger than estimated from total erythrocyte volume and packed cell volume (PCV, large vessel hematocrit). Thus the hematocrit as calculated from the measured plasma and erythrocyte volumes is about 90% of PCV. Since blood in large vessels constitutes a significant fraction of the total blood volume, the hematocrit in some organs must be lower than the total body hematocrit. However, it is only in some minor parts of the circulation that dynamic hematocrits have been found to be smaller than that of the whole body.

The extra plasma volume has been explained in 2 different ways. When plasma volume is estimated, extravascular spaces will be included in organs with discontinuous capillary endothelium, as for instance the liver. The other explanation is based on the Fåhræus-Lindqvist effect. This implies that with the axial streaming of erythrocytes, the small vessels as at any moment contain relatively less erythrocytes than plasma when compared to large vessels. Accordingly, in organs with a large proportion of small vessels, as for instance the lungs, the dynamic hematocrit should be rather low. In accordance with this

It should be noted that during physiological conditions, only end-plate receptors or receptors mediating PTP in fast mammalian muscles are active. PTP in fast muscles begin to develop at 1 Hz and is maximal after about 10 Hz, post tetanic repetition in slow muscle such as the soleus muscle, is only seen after stimulation at 400 Hz which is an unphysiological stimulation frequency. The present results show that PTP in the rat masseter *mus.* is mediated via the activation of nicotinic receptors which have different properties in comparison with the end plate receptors, they are "nondifferentiated" i.e. they are inhibited by both d-tubocurarine and hexamethonium. Since these nicotinic receptors are activated by physiological stimulation frequencies (1 Hz) there evidently exists an additional synaptic mechanism in neuromuscular transmission in fast mammalian muscles. The possibility exists that activation of presynaptic nicotinic receptors are of importance for stimulation above 1 Hz in rat masseter muscle.

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Rabbit lung plasma and erythrocyte volumes. Lung hematocrit in relation to total body hematocrit

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Abstract

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The total body hematocrit has been reported to be 85-90% of packed cell volume (PCV) in several species. We have found smaller values in rabbits. An "extra-plasma volume" must exist somewhere in the vascular system to explain this observation. We have looked for such an extra-plasma volume in the pulmonary circulation. The dynamic hematocrit, as estimated in isolated, perfused rabbit lungs from distribution volumes for plasma and erythrocyte tracers. Estimation was also obtained from indicator-dilution curves and bolus-injections of such tracers avoiding their redistribution. It was then possible to calculate mean transit times for the tracers from their dilution curves directly or applying monoexponential extrapolation from the first part of the downslope of the curves. The dynamic hematocrit of the lung vessels was about 4% of perfused PCV and there was no difference between the results obtained by the different methods. We concluded that in the rabbit only a very small part of the extra-plasma volume is located in the lung vessels. The lung plasma volume is not underestimated by the indicator-dilution technique.

The plasma volume in a mammal is larger than estimated from total erythrocyte volume and packed cell volume (PCV, large vessel hematocrit). Thus the hematocrit as calculated from the measured plasma and erythrocyte volumes is about 90% of PCV. Since blood in large vessels constitutes a significant fraction of the total blood volume, the hematocrit in some organs must be lower than the total body hematocrit. However, it is only in some minor parts of the circulation that dynamic hematocrits have been found to be smaller than that of the whole body.

The extra-plasma volume has been explained in 2 different ways. When plasma volume is estimated, extravascular spaces will be included in organs with discontinuous capillary endothelium, as for instance the liver. The other explanation is based on the Fåhræus-Lundquist effect. This implies that with the axial streaming of erythrocytes, the small vessels will at any moment contain relatively less erythrocytes than plasma when compared to large vessels. Accordingly, in organs with a large proportion of small vessels, as for instance the lungs, the dynamic hematocrit should be rather low. In accordance with this

Aarseth (1971) using a direct method found a very low dynamic lung hematocrit in man. On the other hand indicator-dilution studies on larger animals and man have not shown any large extra plasma volume in this organ (Rapaport *et al.* 1956, Brigham, Wootter and Staub 1973). This discrepancy could, however, be due to a systematic error involved in the monoexponential extrapolation used on the indicator concentration curve in these studies. Thus plasma volume in the lungs might behave as a two-compartment system.

In order to obtain more information on this problem we have used the indicator-dilution technique in isolated perfused rabbit lungs. Mean transit time for plasma and erythrocytes could then be calculated using both monoexponential extrapolation from the first part of the down-slope, and from the complete indicator curve as it was found with recirculation. Any systematic error in the analysis of the plasma or erythrocyte data should then be detected. The dynamic hematocrit calculated from the mean transit times was compared with that found from the distribution volumes of plasma and erythrocyte tracers.

Methods

Experiments were performed on 14 rabbits. In 3 rabbits (b.wt. 2.8–3.1 kg) total plasma and erythrocyte volumes were estimated. 11 rabbits (b.wt. 2.8–3.5 kg) were used as donors for isolated perfused lung preparations. 1–3 of these lungs we measured the plasma and erythrocyte volume from dilution volumes of ^{125}I -serum albumin and ^{51}Cr -labelled erythrocytes. As a second method for erythrocyte volume determination we also measured the hemoglobin content in one of these lungs. In the remaining 8 lungs we performed a dilution test, by simultaneous bolus injections of ^{125}I -serum albumin and ^{51}Cr -erythrocytes.

Total blood volume and total body hematocrit. 3 ml rabbit erythrocytes were incubated with 0.3 μCi $\text{Na}_2^{51}\text{CrO}_4$ and washed 4 times in heparinized saline (Aarseth 1970). The labelled erythrocytes were suspended in saline containing 15 μCi ^{125}I human serum albumin (IFA, Kjeller, Norway). The 3 rabbits were anesthetized by i.v. injections of alphaxolon and tithadolon-acetate (Alfathesin[®], Glaxo), and catheters were inserted in a femoral vein and in an artery. About 70 min after the animals had recovered from anesthesia a weighed amount of the isotope-mixture was given i.v. Arterial blood samples were taken 12 and 18 min thereafter. PCV was found by centrifugation in an International microcapillary centrifuge (Model MB), and the radioactivity in the blood samples and in a diluted sample of the isotope mixture (1:250) were counted in a channel Auto-gamma scintillation spectrometer (Model 220, Packard Instruments Co.). From the 3 observations of albumin distribution volume obtained the plasma volume in each animal was estimated from extrapolation to zero time. The mean of the 3 values for erythrocyte volume was used as a measure for the erythrocyte volume. Total body hematocrit was calculated: erythrocyte volume/erythrocyte volume + plasma volume.

Perfusion of the isolated lungs with homologous blood was carried out as described by Haage, Lee and Waaler (1966) and the system is schematically shown in Fig. 1. The blood was pumped at constant flow from a reservoir kept at 37°C using a Harvard roller pump. The flow ranged between 139–328 ml/min in individual experiments, adjusted to give a pulmonary arterial pressure of about 20–25 mmHg. Papaverine was added to the perfusate (3 mg/100 ml) to prevent vasoconstriction. An electromagnetic flow-meter (Nycotron) was connected to the inlet cannula, as was also a pressure transducer (Statham P23De). Inlet flow and inlet pressure were continuously recorded. The left atrial pressure was kept at 5 mmHg throughout the experiment. The weight of the preparation was continuously recorded (Hewlett Packard force transducer FTA 100-1). Positive pressure ventilation was performed with a Starling pump, the inflation pressure was kept at 7 cm H₂O.

Dilution volumes for ^{125}I -albumin and ^{51}Cr -erythrocytes in the lung. In these experiments the volume of circulating blood was kept as small as possible, about 150 ml. Silk snares were placed around the pulmonary artery and left atrial appendage. When perfusion pressure and preparation weight had stabilized, ^{51}Cr -erythrocytes and ^{125}I -albumin were added to the reservoir. 8 min later the snares were suddenly closed. The lungs were then immersed in liquid nitrogen, and the lung tissue was removed, crushed and homogenized. The hemoglobin content in the lung tissue was estimated by the

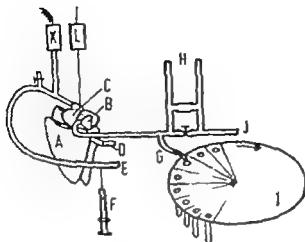


Fig. 1. Schematic drawing of the perfusion system. The lungs (A) were perfused through cannula inserted through the right ventricle (C) into the pulmonary artery (flow pressure as recorded from cannula transducer (K)). The outflow from the left atrium (B) is diverted through a ladder (H) as to sense the pressure at the sampling site. The lungs were ventilated by positive pressure using pump connected to the trachea (D). The lung is kept a-erobic as continuously recorded in force transducer (L). Also injections were performed via syringe-pistol (F) into the pulmonary arterial tube (E). A constant fraction of the outflow was diverted (through G) to the split fraction sampling device (M).

et al. (1965). The plasma and erythrocyte volumes in the lung are calculated from the radioactivity in the vials and in samples of the perfusate. The erythrocyte content of the lung is also calculated from the hematocrit content of the vials and in the perfusate.

Indicator-dilution test. Bolus injections of ^{125}I -albumin and ^{51}Cr -erythrocytes into the arterial cannula and continuous sampling from the outlet were carried out. PCV of the aspirates is adjusted to be close to that of the perfusate. A syringe pistol giving constant volume at constant speed is used for the injections. The sampling device described by Nicolayson (1971) allowed split fractions to be sampled from the outflow every 0.67 or 1.16. Only part of the outflow is sampled, the remaining is discarded and thus recirculation of tracers did not occur. Flow through the system at the time of sampling was taken as the mean volume per unit time of collected outflow immediately before and after the bolus injection. Flow through the sampling catheter is found to scatter at

Since the flow was constant, the volume of the individual sample is nearly the same. The total ^{125}I and ^{51}Cr -activity in each sample were counted. From these data, mean transit times for plasma and erythrocytes were calculated.

The following experiments are performed: Bolus injections of ^{125}I -albumin are given in the perfusion system with no lung in the circuit, and the volume of the tubing was calculated from flow and mean transit time for the tracer. Simultaneous bolus injections of ^{125}I -albumin and ^{51}Cr -erythrocytes were also given, and the mean transit times for plasma and erythrocytes through the tubing are compared.

Bolus injections are performed in lung in the perfusion circuit. In each experiment 2-4 injections were given. In 2 experiments with 8 injections, the transit times for plasma and erythrocytes through the tubing and through the sampling catheter are known. In these experiments the mean transit times through the lung were proper and thus their volumes could be calculated.

The dilution curves from these 2 experiments and from 11 similar tests in 6 other experiments were analyzed in 2 different ways

$$\bar{t} = \sum_{i=1}^n (cpm_i \cdot t_i) / \sum_{i=1}^n cpm_i \quad (1)$$

here \bar{t} is the mean transit time, cpm_i is the radioactivity in sample i , t_i the time from injection to that particular sample, n the number of samples in the series, n is usually about 55.

Aarseth (1971) using a direct method, found a very low dynamic lung hematocrit. On the other hand indicator-dilution studies on larger animals and man have not detected any large extra plasma volume in this organ (Rapaport *et al.* 1956, Brigham, Wooten and Staub 1975). This discrepancy could, however, be due to a systematic error involved in the monoexponential extrapolation used on the indicator concentration curve in these studies. Thus plasma volume in the lungs might behave as a two-compartment system.

In order to obtain more information on this problem we have used the indicator-dilution technique in isolated perfused rabbit lungs. Mean transit time for plasma and erythrocytes could then be calculated using both monoexponential extrapolation from the first part of the down-slope, and from the complete indicator curve as it was found after recirculation. Any systematic error in the analysis of the plasma or erythrocyte curves should then be detected. The dynamic hematocrit calculated from the mean transit times was compared with that found from the distribution volumes of plasma- and erythrocyte tracers.

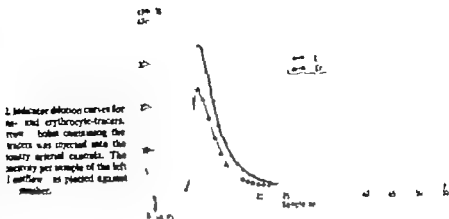
Methods

Experiments were performed on 14 rabbits. In 3 rabbits (b.wt. 2.8–3.1 kg) total plasma and erythrocyte volumes were estimated. 11 rabbits (b.wt. 2.8–3.5 kg) were used as donors for isolated perfused lung preparations. In 3 of these lungs we measured the plasma and erythrocyte volume from dilution studies of ^{125}I -serum albumin and Cr-labelled erythrocytes. As a second method for erythrocyte volume estimation we also measured the hemoglobin content in 2 of these 11 lungs. In the remaining 8 lungs we performed 3 indicator dilution tests, by simultaneous bolus injections of ^{125}I -serum albumin and ^{51}Cr -erythrocytes.

Total blood volume and total body hematocrit. 3 ml rabbit erythrocytes were incubated with 65 μCi $\text{Na}_2^{51}\text{CrO}_4$ and washed 4 times in heparinized saline (Aarseth 1970). The labelled erythrocytes were suspended in saline containing 15 μCi ^{125}I -human serum albumin (TFA, Kjellev, Norway). The 3 rabbits were anesthetized by i.v. injections of lphaxolon and alphadolon-acetate (AH 1000, Glaxo), and catheters were inserted in a femoral vein and in an artery. About 20 min after the animals had recovered from anesthesia a weighed amount of the isotope-mixture was given i.v. Arterial blood samples were taken 12 and 18 min thereafter. PCV was found by centrifugation in an international microcapillary centrifuge (Model MB) and the radioactivity in the blood samples and in a diluted sample of the isotope mixture (1:250) were counted in a 2 channel A to-gamma scintillation spectrometer (Model 2220, Packard Instruments Co.). From the 3 observations of albumin distribution volume obtained the plasma volume in each animal was estimated from extrapolation to zero time. The mean of the 3 values for erythrocyte volume was used as a measure for the erythrocyte volume. Total body hematocrit was calculated as erythrocyte volume/erythrocyte volume + plasma volume.

Perfusion of the isolated lung with homologous blood was carried out as described by Hargreaves and Waaler (1966) and the system is schematically shown in Fig. 1. The blood was pumped at constant flow from a reservoir kept at 37°C using a Harvard roller-pump. The flow ranged between 139–320 ml/min in individual experiments, adjusted to give a pulmonary arterial pressure of about 20–25 mmHg. Papaverine was added to the perfusate (3 mg/100 ml) to prevent vasoconstriction. An electromagnetic flow-probe (Nycotron) was connected to the inlet cannula, as was also a pressure transducer (Statham P23De). Mean flow and inlet pressure were continuously recorded. The left atrial pressure was kept at 5 mmHg throughout the experiment. The weight of the preparation was continuously recorded (Hewlett Packard force transducer FTA 100-1). Positive pressure ventilation was performed with a Starling pump, the inflation pressure was kept at 7 cm H_2O .

Dilution volumes for ^{125}I -albumin and ^{51}Cr -erythrocytes in the lung. In these experiments the volume of the circulating blood was kept as small as possible, about 150 ml. Silk snares were placed around the pulmonary artery and left atrial appendage. When perfusion pressure and preparation weight had stabilized, ^{51}Cr -erythrocytes and ^{125}I -albumin were added to the reservoir and 8 min later the snares were suddenly closed. The lungs were then immersed in liquid nitrogen, and the lung tissue was removed, crushed and homogenized. The hemoglobin content in the lung tissue was estimated by the method described by



and 0.7 ml, respectively and the dynamic hematocrits in % of PCV of perfusate were 94.6 and 95.0. The last lung in this series was perfused with pulsatile flow. This made difference as regards plasma excess or pulmonary blood volume (Table II).

The volume of the tubings in the perfusion system was found to be 20.15 ml (median, range 7-30.3). The ratio between the transit time for ^{125}I -albumin and that of ^{51}Cr -erythrocytes from bolus-injections was estimated to 8 bolus-injections. The median value was 1.004 (range 0.55-1.040).

Fig. 2 shows a set of indicator-dilution curves obtained in one of the perfused lungs. The mean transit times for plasma and erythrocytes were calculated from 8 injections, and the flows are corrected for delay in the tubings and in the sampling catheter. The results are shown in Table III. The median value for dynamic hematocrit was 93.8 % of perfusate CV (range 90.5-95.0) and thus identical with that found with the direct method. The plasma excess in the lungs were 1.25-2.0 ml (median value 1.55 ml). The data from the 23 bolus-injections of ^{125}I -albumin and ^{51}Cr -erythrocytes were analyzed in different ways as

TABLE III. Mean transit times for plasma and erythrocytes in perfused rabbit lungs. Plasma- and erythrocyte-volumes were calculated from flow, PCV of perfusate and the transit times. Excess plasma = the difference between the plasma volume and that calculated from erythrocyte volume and PCV. It is also calculated as % of actual lung blood volume.

	Flow ml/min	PCV of perfusate	t_{plasma}	t_{eryth}	Lung plasma ml	Lung eryth. ml	Excess plasma ml	Excess plasma %
Exp. 16-9								
1	140	34.7	7.3	6.3	19.6	8.8	1.9	6.5
2	144	35.3	7.3	6.75	19.3	9.7	1.6	3.5
3	146.5	35.8	7.3	6.7	19.2	9.9	1.5	3.2
Exp. 21-9								
1	208	28.3	6.4	5.75	15.8	5.75	1.25	5.8
2	198	30.3	6.6	5.7	15.1	5.7	2.0	9.6
3	196	30.7	6.9	5.3	13.4	5.3	1.4	7.5

TABLE I Distribution volumes for ^{125}I human serum albumin and ^{51}Cr -labelled rabbit erythrocytes. Plasma excess is the difference between the measured plasma volume and that calculated from erythrocyte volume and PCV. It is also given as % of total blood volume.

	Rabbit no.		
	1	2	3
Body weight, kg	3.1	3.0	2.8
Plasma volume, ml/kg b.wt.	32.8	34.2	33.4
Erythrocyte volume, ml/kg b.wt.	15.0	15.5	14.0
PCV	35.5	34.6	34.3
Plasma excess, ml/kg b.wt.	5.3	4.7	6.5
Plasma excess, %	11.1	9.5	13.7

) From the 5 first samples on the downslope of the indicator curve, the best fit (least squares derived) monoexponential curve was found. The measured values for cpm_t were replaced with values taken from this monoexponential curve and \bar{t} was calculated as for 1).

3) All samples on the downslope part were used to find the best fit bi-exponential curve (least squares deviation). cpm_t values were read from this curve and used in the formula given in 1). The calculation was also extended to infinity. The best fit analysis was done on a Nord 10 computer (Norsk Data).

Results

The results for body plasma and erythrocyte volumes are given in Table I. The estimated plasma volumes were 14, 16 and 18 ml respectively larger than could be expected from the measured erythrocyte volumes and PCV. Total body hematocrits thus were 88.7, 90.8 and 85.5% of PCV respectively in the 3 animals.

Table II shows the results from the 3 lungs in which plasma and erythrocyte volumes were determined from the distribution volumes of tracers. The erythrocyte volume was determined in all 3 from the ^{51}Cr -erythrocyte dilution volume, in two of them also from the hemoglobin content of the lung tissue. As is seen in Table II the two methods for determination of erythrocyte volume gave nearly identical results. The plasma excess was 11.1, 9.5 and 13.7% respectively in the 3 animals.

TABLE II Plasma volume as estimated in isolated perfused rabbit lungs from distribution volumes of ^{125}I human serum albumin. Erythrocyte volumes are found from the lung content of ^{51}Cr -labelled erythrocytes or hemoglobin. Plasma excess is calculated as difference between measured plasma volume and that calculated from PCV and erythrocyte volume.

	Rabbit no.		
	4	5	6
Body weight of donor rabbit, kg	3.15	3.15	3.40
Lung plasma volume, ml	9.29	8.6	10.08
Lung erythrocyte volume, ml	4.50	4.13	4.82
^{51}Cr -erythrocytes	5.19	4.49	
Hemoglobin	35	35.8	34
PCV of perfusate	1.0	0.7	0.7
Plasma excess in lung, ml	7.3	5.8	4.7
Plasma excess in lung, %			

olumes of plasma and erythrocyte tracers in rapidly frozen animals. The dynamic hematocrit found has almost invariably been significantly smaller than the total body hematocrit (Aarneth 1971). This is in contrast to the results obtained in larger animals with more indirect methods referred to above. This discrepancy could be explained if there is a second plasma compartment in the lungs, undetected by the indicator-dilution technique when monoexponential extrapolation is used to circumvent the recirculation. In the present expts. on isolated, perfused lungs, we were able to avoid the monoexponential extrapolation. It turned out that mean transit times calculated from extrapolated curves were shorter than the observed transit times. Most of this difference must be due to the perfusion system as it also was found in model expts. with no lung in the circuit. It remained a small but insignificant difference which was slightly larger for the plasma than for the erythrocyte transit time, this would hardly make any difference as regards the pulmonary dynamic hematocrit.

The indicator dilution curves thus did not have a monoexponential downslope. Even in a two-compartment system, however, the indicator dilution curve will end in a monoexponential tail (Lassen and Sejrsen 1971). By ending the sampling before this tail is reached, mean transit time calculated from extrapolation would be seriously underestimated (Lassen and Sejrsen 1971). In the present experiments, the median concentration of last sample was 0.3% of peak concentration (96.5% interval 0.25-0.6). For one curve, extrapolation was done from a sample which indicator concentration was 6% of peak concentration. The mean transit time calculated from this extrapolated curve was negligibly smaller (3%) than the one calculated from the observed points. We therefore suggest that we have reached the monoexponential tail in our expts.

The mean transit times calculated from the observed points were identical with those obtained by integration of a biexponential curve fitted to the observed points. The second exponential probably will describe the tail. By integration to infinity of this biexponential curve, the mean transit times increased by only 2.76% and 2.07% for plasma and erythrocytes, respectively. It can be concluded that monoexponential extrapolation from the first part of the downslope does not to any large extent underestimate mean transit time for plasma as compared to that of erythrocytes.

The vascular volume seen by the indicator dilution technique includes the pulmonary artery and left atrium, and will thus be larger than the distribution volume within the lung tissue proper (Table II and III). The latter volume was determined from both ^{51}Cr -erythrocytes and hemoglobin in the homogenized lung, and the two methods differed by only 8 and 13% in the 2 expts. This suggests that the labelled erythrocytes are fairly well distributed, and thus also the labelled albumin.

In conclusion, if monoexponential extrapolation of indicator-dilution curves gives wrong estimates of transit times, then this error will be the same for plasma and erythrocytes. In our isolated lungs, there seemed to be no plasma compartment unseen by the indicator dilution tests. We do not know therefore in which vascular compartment the extra plasma volume is located.

* I would like to thank research fellow Gunnar Sjöden for invaluable help with the data-processing. We also thank Keris Långström for skilful technical assistance.

described in Methods. For each curve the mean transit time was calculated from the observed concentrations of indicators throughout the sampling period. This value was set to 100 %. Using monoexponential extrapolation from the first 5 samples on the downslope we found the median value for the mean transit time of erythrocytes was 90.01 % (91% confidence interval 87.90-92.32) while for plasma it was 88.88 % (86.82-90.41). The difference between the mean transit times obtained from the observed curve and from monoexponential extrapolations were also seen in the model experiments with no leak in the circuit (median values 91.57 % and 91.64 % for erythrocytes and plasma respectively).

The analysis in which all the points on the downslope were fitted to a biexponential curve and mean transit times calculated from the integral up to the time of the last sample gave the following results. The median value for plasma was 99.89 % (99.63-100.08) while for erythrocytes was 99.88 % (99.51-100.32). These biexponential curves were also integrated to infinity; the mean transit times calculated then were 102.76 % (101.51-104.76) and 102.77 % (100.74-105.07) for plasma and erythrocytes, respectively.

Discussion

The median value for total blood volume in 3 rabbits was 47.8 ml/kg b.wt., which is in the lower normal range (Little 1970). The total body hematocrit was between 85-90 % PCV; the same order of magnitude is found in man and dog (Gregersen and Rawson 1964) and cat (Aarseth and Bo 1972) and rat (Aarseth 1970).

The dynamic hematocrit in different tissues can be calculated from dilution curves using both plasma and erythrocyte tracers. With Bradley's equilibration method, Larsen and Lassen (1964) and Larsen (1966, 68) measured the dynamic hematocrit in the cerebral vessels, in the lungs and in the lower extremity. In all these organs they found dynamic hematocrits which were larger than total body hematocrit. Similar results have been obtained by Crane *et al.* (1960) who applied the Stewart-Hamilton technique on the same organs. Rapaport *et al.* (1956) and Brigham, Woolverton and Staub (1975) used the same method on lungs in dogs and sheep and reached the same conclusion. Larsen, Tyystjärvi, Winkler (1963) applied Bradley's method on the splanchnic circulation and measured a dynamic hematocrit in this area that equalled total body hematocrit. In patients with portacaval anastomosis they were able to study the liver vessels separately and revealed a dynamic hematocrit of only 86% of total body hematocrit (Larsen, Winkler and Tyystjärvi 1963). Aarseth, Aarseth and Bergan (1976) measured plasma and erythrocyte distribution volumes in rat liver. Only in animals with bile duct occlusion was the liver dynamic hematocrit smaller than the total body hematocrit. In control animals it was slightly larger. Studies on the renal circulation with the Stewart-Hamilton method have revealed a dynamic hematocrit that is similar to that of the total body hematocrit (Chinard, Enos and Nolan 1964), while direct estimates of distribution volumes have unveiled a slightly smaller hematocrit in this organ (Rasmussen 1973). Thus so far the excess plasma volume known to exist has not been found in any of the organs studied.

In our laboratory the dynamic hematocrit of the rat lung has been evaluated from distrib-

Maintenance of lactose secretion during acute insulin deficiency in lactating goats

By

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Abstract

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Injection of alloxan diabetes in 5 lactating goats resulted in reduced milk yields in 3 of the animals. Lactation yield was unchanged in two. After treatment of the diabetic goats with insulin for 4-5 days—the last 4 days continuously—lactose secretion returned to the control values before alloxan administration provided normoglycaemia developed. In experiments infusion of large doses of insulin caused hypophosphataemia.

20-30 per cent reduction in lactose secretion rates. In the course of 1 h after withdrawal of the insulin infusion, partial signs of insulin deficiency developed as evidenced by steadily increasing plasma glucose concentrations. Nevertheless, lactose secretion continued at the same rate as during insulin infusion. The 4 h studied after discontinuation of the insulin infusion the goats were lactose secretion increased due to insulin-induced hypophosphataemia, lactose secretion returned to control values best following discontinuation of insulin infusion the plasma glucose concentrations increased into normal and diabetic ranges. It is concluded that during insulin deficiency of short term duration, mammary lactose secretion continued at normal rate. Since lactose is the major product of mammary glucose utilization, it is stated that glucose uptake at the mammary gland was not reduced by short term insulin deficiency.

Lactation is a process which proceeds at a nearly constant rate throughout the day irrespective of changes in absorption of metabolites from the digestive tract and irrespective diurnal variations in the plasma concentrations of hormones (e.g. insulin) regulating the disposal of energy substrates. In lactating ruminants a considerable amount of the available energy substrates is used for milk synthesis. Thus, the glucose uptake of the mammary gland amounts for 80-85 per cent of the glucose utilization in lactating dairy ruminants. Two thirds or more of the mammary uptake is converted to lactose (Ammon and Linzell 1964, Ikertallie *et al.* 1974). An important question therefore arises. Is glucose uptake by mammary glands influenced by plasma insulin concentrations in a similar way as glucose uptake in peripheral insulin-sensitive tissues? Since the synthesis of lactose by the mammary gland accounts for the major part of the mammary glucose uptake in dairy ruminants, any major changes in glucose uptake might also result in altered rates of lactose secretion.

The question can only be answered partly: Insulin is in fact essential for the maintenance of secretory activity in mammary tissue both in rodents (Walters and McLean 1968, Martin

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its separated sides (5 U/ml) between samplings. Ten pairs of arterial and venous blood samples taken every hour 20 and 30 min after each oxytocin injection.

Anal procedures

Plasma was measured spectrophotometrically by glucose oxidase method (Boehringer reagents). Measurements were run in duplicate and the standard deviation of duplicates in the normal plasma range (60–160 mg/100 ml) as 1 mg/100 ml and in the diabetic range (200–300 mg/100 ml) between 4–6 mg/100 ml.

Acetoacetic acid was measured spectrophotometrically by an automated modification of the Strecker reaction as dialysis of plasma (Blom and Hult 1973).

Free fatty acids were measured by the radio-active nickel binding method of Ho (1970) with modifications.

Milk urea was measured by radioimmunoassay using electron coated charcoal to separate bound from unbound (Forstner and Forstner 1969). Bovine plasma was used both as standard and for the assay of anti-bovine antibodies. An antibody concentration giving maximum sensitivity in the range of urea concentrations was chosen. The samples were measured in duplicate. The variation across varied between 17% in the lower range (0.2–0.6 mg/ml) and 7% in the higher range of plasma urea concentrations. The lower limit of detection usually about 0.1 mg urea/ml in plasma.

Milk lactose was measured spectrophotometrically as glucose (by the glucose oxidase reaction) after milk hydrolysis by 1.5 U per 5 ml of *E. coli* β -galactosidase (Boehringer). The milk first diluted and proteins precipitated (Cheeser 1943). A sample containing 50–100 μ g lactose is incubated with 5 ml of glucose oxidase reagent (Boehringer) at 37°C for 45 min. Absorbances were measured on Beckman DU spectrophotometer at 420 nm. Analytical grade lactose was used as standard. The relationship was obtained between absorbance and lactose concentration within the actual range. Milk samples were analysed in duplicate. The coefficient of variation on duplicates is between 2 and

Results

Clear changes in the metabolism were evident 48 h after alloxan injection. Low plasma insulin and high plasma glucose concentrations (Table I) were accompanied by increased concentrations of plasma free fatty acids and acetoacetic acid (from 0.24 to 0.55 and from 0.04 to 0.19 mM on the average, respectively). At this time of the experiment lowered milk yields were noted in 3 of the goats (from 20–75 per cent of the control yields), while the yields in the 3 others were essentially unchanged.

In the 4 experiments where normal plasma glucose concentrations were obtained after the insulin infusion the hourly lactose yields were approximately identical to those observed in control experiments immediately before alloxan administration (Table II).

When the infusion was discontinued a sharp rise in blood glucose levels was observed in all goats. During the 4 h observation period, plasma glucose concentrations of between

Table I. Changes from control to alloxan diabetic state. Control. Average and S.D. of plasma concentrations in 10 arterial samples taken during 5 h period two consecutive pre-allowan lactose secretions. Alloxan average and S.D. of 5 arterial samples taken at hourly intervals starting 48 h after alloxan administration.

Insulin μ g/ml		Glucose mg/100 ml	
		Control	Alloxan
0.52 \pm 0.12	0.27 \pm 0.03	65.9 \pm 4.2	295 \pm 6
2.00 \pm 0.30	0.15 \pm 0.03	49.1 \pm 3.3	256 \pm 15
0.67 \pm 0.20	0.25 \pm 0.08	75.8 \pm 3.1	265 \pm 21
0.92 \pm 0.17	0.23 \pm 0.02	80.0 \pm 6.1	305 \pm 7
0.96 \pm 0.06	0.10 \pm 0.01	63.6 \pm 2.0	308 \pm 11

and Baldwin 1971 *a*, *b*) and goats (Nowak and Działoszynski 1967). Lack of insulin in the rat for 36–48 h or more reduces milk secretion and enzymatic activity in the mammary gland. Alloxan diabetes of some weeks duration causes complete and irreversible loss of secretory activity in lactating goats. These changes are probably related to the deterioration of metabolic machinery of the mammary cells, and do not give information as to the physiological regulatory role of insulin in mammary carbohydrate uptake and secretion. While the long- or short-term effects of insulin deficiency in the rat include reduced glucose oxidation, fat, lactose and casein synthesis (Baldwin and Louis 1975) comparable information in ruminants is lacking. Diabetes mellitus is a very rare disease in ruminants and has not been reported during lactation (Phillips *et al.* 1971). Therefore, to study the short-term effects of insulin deficiency lactating goats were made diabetic by administration of alloxan. Mammary lactose secretion was measured in two situations. After a 24 h *i.v.* infusion of insulin to normalize the metabolism, and subsequently for four hours after the infusion was discontinued. During this last period marked metabolic signs of insulin deficiency develop.

Animals

5 adult goats weighing 30–45 kg were used for the present study. The animals were in second to sixth year of lactation and yielded from 0.9–2.0 kg milk daily. The goats were housed indoors in individual pens during the experiments and fed hay and a pelleted concentrate ration *ad lib.* Food was given twice daily about 8 a.m. and 3 p.m., and milking was performed in connection with the meals. Feeding and milking were carried out as usual on days when experiments were to be performed. The goats were allowed to graze undisturbed for $\frac{1}{2}$ h before the experiments started. Some weeks prior to the experiments the carotid artery was exteriorized into a skin-covered loop to facilitate the sampling of arterial blood.

Methods

Experimental design

Alloxan diabetes was established by a single injection of D-allylamine oxalate (D-allylamine oxalate) at a dose which has previously been effective in goats and sheep (50 mg/kg b.wt., Nowak and Działoszynski 1967; Filbert *et al.* 1969). Blood samples were taken 48 h after alloxan administration and plasma concentrations of insulin, glucose, free fatty acids and acetone were measured in order to verify the presence of a diabetic condition. The animals were stabilized with insulin for 4–5 days. Lactose secretion was measured in two experimental series—treated alloxan diabetes and insulin deficiency—and compared with values obtained once to twice daily after alloxan administration.

1) *Insulin-treated alloxan diabetes.* Insulin treatment was started 48 h after alloxan administration. Insulin (porcine) was injected subcutaneously for 3–4 days and subsequently infused *i.v.* for 4 h. During the infusion insulin diluted with saline was given through a jugular catheter at a rate of 1.7 to 2.0 µg/kg (4.6 ml/h) the aim being to achieve a normal plasma glucose level. After at least 70 h (usually 4 h) of insulin treatment lactose secretion was measured for 2 h. In 3 goats approximately normal plasma glucose levels were observed while in 2 hypoglycemic glucose values (35–45 mg/100 ml) were obtained at the end of the insulin infusion. In one of the latter a lower dose of insulin was infused 2 days later. On this occasion normoglycemia was maintained throughout the last part of the infusion. Consequently 4 experiments with normal and two with hypoglycemic glucose levels are reported.

2) *Insulin deficiency.* The infusion of insulin was discontinued after 2 h of milk and blood sampling. Changes in plasma glucose concentration and lactose secretion were then studied for 4 h during development of a diabetic condition.

Milking. Intravenous injection of 100 mU of synthetic oxytocin was given to evoke milk ejection (Lind 1960). 3 min elapsed from oxytocin administration to the completion of milking. The goats were milked by hand and the volumes from the two udder halves were measured separately.

Blood sampling. Catheters for blood sampling (o.d. 1.0 mm) were inserted into the carotid artery loop under local anesthesia. Plasma was separated, frozen and kept at -70°C until analysed. The catheters were

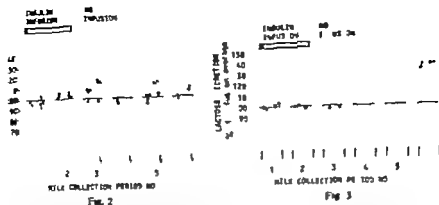


Fig. 2

Fig. 3

2. Lactose secretion during and after the infusion of insulin to 4 alloxan diabetic goats. Lactose secretion is per cent of the average secretion for each gland during the 2 h infusion period. R and L. Right and left respectively. Individual symbols.

3. Lactose secretion during and after the infusion of insulin to 2 alloxan diabetic goats. Due to the use of a too large dose of insulin hypoglycaemia developed at the end of the infusion (see Fig. 1). Legend as in Fig. 2.

one secretion varied somewhat, but no significant changes were observed (Fig. 2). In the 2 expts. with hypoglycaemic goats lactose secretion was increased by 30–40 per cent following cessation of insulin infusion (Fig. 3). During the infusion period lactose secretion was 20–30 per cent lower than that recorded in the control expt. before alloxan administration (Table II). Thus the increase observed after insulin withdrawal in these goats actually seems to have been a 'normalisation' of lactose yield which was depressed by hypoglycaemia during the insulin infusion.

Discussion

The changes in plasma metabolite concentrations after alloxan administration were comparable with those observed in sheep by Jarret *et al.* (1974), and indicate that the amounts of insulin secreted by the pancreas 48 h after alloxan administration were insufficient to maintain normal fat and carbohydrate metabolism. Insulin was given to the alloxan diabetic goats for 4–5 days to allow time for a normalization of the diabetic metabolism before the lactose secretion measurements were carried out. The finding that lactose was secreted at approximately the same rate as in the control situation at the end of the 24 h insulin infusion, indicates normal metabolism at this time of the experiment.

After discontinuation of the insulin infusion plasma insulin concentrations declined rapidly. But the insulin deficiency was not associated with reduced lactose secretion despite the fact that patent signs of a diabetic metabolism developed during the last 2–3 h of the observation period. The rise in lactose yields (by 30–40%) after the cessation of insulin infusions in the 2 expts. with hypoglycaemic animals further substantiates this. In spite of the insulin deficiency lactose secretion in the 2 hypoglycaemic goats increased to the levels observed before the alloxan injections when plasma glucose returned to normal or hyperglycaemic levels.

The importance of insulin as a major regulator of energy metabolism in ruminants has

TABLE II Lactose secretion rate in the control experiment before alloxan administration and after termination of the alloxan diabetes by a 4 h insulin infusion. Before: average and S.D. of 3 test periods. Insulin treated diabetes: average of one hour periods at the end of a 4 h insulin infusion. R and L: Right and left mammary gland.

Goat	Before alloxan		Insulin treated diabetes	
	R	L	R	L
B	70.1 \pm 1.7	19.9 \pm 0.9	21.6	22.6
C	34.1 \pm 3.2	34.8 \pm 4.5	34.1	33.0
D	25.9 \pm 2.0	30.1 \pm 3.2	23.9	26.7
E	23.4 \pm 1.1	22.5 \pm 0.8	18.0	18.4
M	20.3 \pm 1.2	17.9 \pm 1.0	1.6	19.7

Hypoglycemia due to the infusion of a too large dose of insulin.

300 and 500 mg/100 ml were reached in most of the animals (Fig. 1). Plasma insulin concentrations decreased within 20 min after the discontinuation of the insulin infusion (Fig. 2). Insufficient time elapsed so as to allow insulin to fall to the low levels seen in normal alloxan diabetes (Table I). The high plasma glucose concentrations indicate a defective metabolism, however. In the 4 goats with initially normal plasma glucose concentration

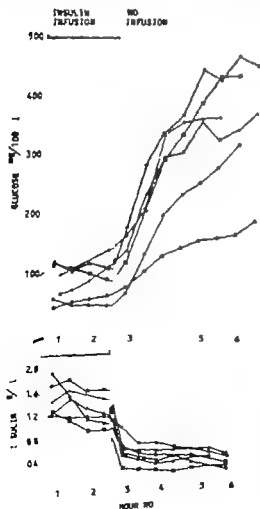


Fig. 1 Plasma glucose and insulin concentrations during and after the 4 h infusion of insulin in alloxan-diabetic goats. 6 different curves are shown. The symbols \circ - \circ and Δ - Δ refer to the 4 goats during infusions which gave hypoglycemic and normal plasma glucose concentrations respectively.

Technical assistance of M. Per Macra is acknowledged. The study was supported by the Norwegian Agricultural Research Council.

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been definitely settled (Review Basset 1975). Considerable diurnal fluctuations in plasma insulin concentrations have been observed in various physiological states (Lactation F and Blom 1973 Hart *et al* 1975 Pregnancy Hove and Blom 1976). Periods with high insulin concentrations are associated with feeding, while the 24 h minima coincide with periods with low absorption of metabolites from the gastrointestinal tract. The present results demonstrate that the mammary secretion of lactose was in fact unaffected by short term changes in the plasma concentrations of insulin to an extent which profoundly affects the carbohydrate metabolism of other tissues. This constancy in lactose secretion could be explained by the existence of an insulin-independent glucose uptake mechanism in mammary tissue. The mammary cells would then continue to utilize glucose at their normal rate irrespective of the rapidly vanishing plasma insulin concentrations. The arguments for the presence of an insulin-independent uptake mechanism for glucose in the udder rest on the assumption that the half life of insulin action on the transport mechanism is of the same magnitude as in other tissues, *i.e.* well below the 4 h studied in the present experiments referred to earlier involving long term insulin deficiency (Nowak and Dziąszynski 1967 Martin and Baldwin 1971 *a, b*) demonstrate that a continuous secretion of insulin is a prerequisite for milk secretion. The time required to obtain reductions in lactation and milk production in insulin deficient animals (usually in the order of days) is far longer than that required to change the rates of carbohydrate transport in insulin sensitive tissues. Thus, although alternatively insulin might be an active regulator of mammary glucose uptake, most available evidence seems to favour an explanation involving an insulin independent glucose uptake mechanism in the mammary gland. From experiments in rats Martin and Baldwin (1971 *b*) suggested that the primary action of insulin on mammary cells was to regulate intracellular processes, and not the transport of nutrients. Far Hove (to be published) in another series of experiments could not find any effect of insulin on mammary glucose uptake and lactose secretion rate in goat mammary glands removed from blood with artificially increased arterial plasma insulin concentrations.

The pronounced rise in arterial glucose concentrations after insulin withdrawal could be the effect of changes in both hepatic and extrahepatic carbohydrate metabolism. The effect of insulin on the glucose metabolism of ruminants does not seem to take up significant amounts of glucose (Review Blaxter *et al* 1969) but the rate of glucose release is strongly influenced by insulin (West Passey 1967) and by glucagon (Basset 1971 Clark *et al* 1976). Although plasma glucose concentrations were not measured in the present investigation, reduced insulin/glucose ratios favouring hepatic glucose release would be the consequence of insulin withdrawal. Reduced glucose consumption in peripheral tissues during insulin withdrawal could contribute to the glucose rise, although the quantitative importance seems unclear at present time (Cabill 1971 Jarret *et al* 1974).

In conclusion, the present study demonstrates that in the dairy goat a normal lactation can be maintained during periods of insulin deficiency which are sufficiently long to cause great changes in the carbohydrate metabolism of the animal. In contrast to a more prolonged lack of insulin (for days or weeks) will invariably lead to drastic irreversible decreases in milk production as demonstrated in the goat by Nowak and Dziąszynski (1967).

thus evident that the epaxial muscles form an integral part of the locomotor mechanism. Few data are available about their physiological properties and reflex interactions. In neurophysiological investigations of the longissimus and multifidi muscles in the lumbar back region (Carlson and Lindquist 1976) have shown that these muscles, like those in the leg, are tonically active in decerebrate preparations and that their activity is evoked by exteroceptive stimulation applied to certain skin areas on the trunk and on limbs. The localization of skin areas from which facilitation or inhibition of motor unit activity could be evoked prompted speculations about the contribution of back muscles in driftnet reactions of the animal. However for further analyses of extero- and proprioceptive reflex patterns in the lumbar back muscles as well as for studies of their conduction properties it proved necessary to gather additional information about the morphology and innervation of the muscles. Such observations will be presented in the first part of this paper. The structural organization of the lumbodorsal fasciae will also be described. The second section of the paper deals with the contraction properties of the two main muscles longissimus and iliocostalis and the medially located multifidi and interspinales.

Histochemical investigation of these muscles will be presented in a subsequent paper (Carlson 1978).

Material and methods

Morphological observations on the lumbar back muscles were made on about 25 cats also utilized for other purposes. In four cats the individual muscles were dissected free and weighed. Cross sections of the skin areas were obtained by sawing one crabbed and one frozen preparation. The sections were stained at low magnification (6-16 \times) and photographed. The mobility of the lumbar spine was estimated in X-ray pictures of deeply anaesthetized but otherwise intact animals. The cats were placed lying on one side and manually held in different postures during the exposures.

The contraction properties of the medial muscles multifidi and interspinales and the lateral muscles longissimus and iliocostalis were studied in 14 cats (2.3-5.4 kg) anaesthetized by i.p. injections of Nembutal (1-6 mg/kg b.wt.). Due to the close anatomical relationship between the medial muscles as well as between the lateral muscles the preparation of individual muscle attachments for recording of mechanical forces tends to separate muscle fibers and to interfere with the blood supply. Another factor limiting

possibilities of selective studies is that the various branches of the dorsal roots of the spinal nerves supply individual muscles generally run intramuscularly throughout their course. Hence, preparation of these branches for indirect activation of the muscles might also cause some damage to them. With the present procedure used, as described below, the conclusions drawn in the analyses of the contraction properties of the various muscles should however be reasonably safe.

A midline skin incision was made extending from the upper thoracic to the sacral region and then the skin layers in the lumbar region and the intermuscular domes were cut through bilaterally.

From the multifidi and interspinales muscles at a given level along the lumbar spine recordings are made by connecting the appropriate tendon to the recording instrument leaving the insertion onto the vertebrae intact (cf. Fig. 1). The longissimus and iliocostalis are separated from the iliac bone together with a small segment of the iliac crest which was attached for mechanical recording. A loose connection to the deep fascial layer (cf. Fig. 1, 2) was connected for registration of contractions. Even though in direction of pull is adjusted so as to give maximal contractions the recordings did probably not represent the maximal force of the muscles.

The muscles were indirectly activated by stimulation of their peripheral nerves or the corresponding ventral roots. The former procedure was applied to the multifidi and interspinales which both are supplied by medial branches of the dorsal rami. No attempt was made to separate and selectively stimulate the individual nerves to these muscles. The nerve arising from a given segment was dissected free for stimulation lateral of the superior articular process. For activation of the longissimus and iliocostalis ventral root stimulation was applied after transection of the lumbar spine and transection of the dorsal roots. T

Morphology and contraction properties of cat lumbar back muscles

By

HANS CARLSON

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Abstract

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The gross morphology, innervation pattern and contraction properties of lumbar back muscles in the cat were investigated. The medially located multifidus and interspinales are formed by short bundles connecting adjacent vertebrae. Laterally located bundles composing longissimus and iliocostalis are attached to the spinous processes by a fascial layer and to the pelvic bone by a well developed interosseous septum. Different spinal segments innervate the various sections along the muscles in such a way that the myotomes composing a muscle belly seem to be arranged after one another in a row. The contraction time of a maximal isometric twitch is 3.4 ms for multifidus and interspinales and 29 ms for longissimus and iliocostalis. No large segmental variations of contraction times were found. The time course of twitch responses and a study of reflex contractions in middle and lower sections of lateral muscles indicate the presence of a slowly contracting portion located to the longissimus. The initial muscle length is more decisive for twitch amplitude of medial than of lateral muscles. In medial muscles summation of individual twitches starts at a stimulus frequency of 10-15 Hz and apparent fusion occurs at about 50 Hz. For lateral muscles corresponding values are 15-20 Hz and about 70 Hz. The contractile tension declines markedly in muscles during a 10 min period of stimulation at 5 Hz.

While a considerable amount of information is available about the functional organization of mammalian limb muscles, very little attention has been paid to the muscles primarily responsible for movements and posture of the vertebral column. The significance of the epaxial muscles for locomotion has been considered in studies concerning the mechanics of the tetrapod skeleton (Gray 1944, 1968) and of the vertebral column (Slipper 1946) and in an analysis of the motions of the running horse and the cheetah (Hildebrand 1959). From these works it appears that in lower tetrapods the propulsive drive may have its origin in the vertebral muscles whereas in mammals these muscles provide the trunk with adequate rigidity during locomotion. In addition, certain animals, e.g. the cat, display marked flexion and extensions of the axial skeleton during running (Goslow, Reinking and Stuart 1978). These movements, in part executed by the epaxial muscles, are coordinated with limb movements so that an increase of the stride is achieved.

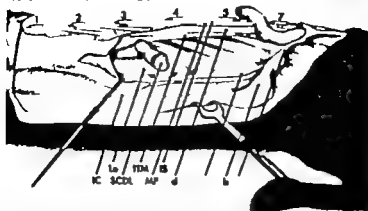


Fig. 1. Dorsolateral view of muscles in the cat's lumbar back region. Lo, longissimus, IC, ilio-costalis, MF, microcaudalis dorsalis lateralis (cut and partly freed from Lo); ITM, intertransversarii mediales multifidi, IS interspinales 1, 5 and 7; spinous processes of lumbar vertebrae; a, iliac crest, b and c, fasciae covering dorsal muscles, cut and retracted to show structure and fiber direction of underlying muscles; d, fascia covering multifidus muscles, cut along dorsolateral aspect of muscles.

the total epaxial muscle mass in the region while the multifidus represent around 10% of the microcaudalis dorsalis lateralis about 8% and the intertransversarii mediales together with the interspinales only a few percent.

Before giving a more detailed description of the muscles it seems pertinent to consider the organization of the various connective tissue structures in the lumbar region.

Three separate fascial layers, attached to the iliac crest, cover the dorsolateral aspect of the back muscles (cut and retracted in Fig. 1). Dorsally they are attached to the spinous processes and the supraspinous ligaments. The thin superficial sheet covers all the muscles and inserts into the transverse processes and the intertransverse ligaments (b in Fig. 1, 2). The inner layer which is more prominent, covers the medial muscles and the dorsolateral aspect of the longissimus and then enters the lateral muscle mass (c in Fig. 1, 2).

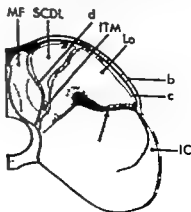


Fig. 2. Cross section at level of 6th lumbar vertebra. Fasciae and muscles marked as in Fig. 1. Darker dotted area of Lo shows localization of muscle tissue appearing red in color. Arrows indicate approximate position of fusion between lateral and medial connective tissue components of intertransverse system.

avoid mechanical disturbances from other muscles the ventral ram of the spinal nerve was cut. Experiments applying ventral root stimulation. For elicitation of reflex contractions of the longissimus iliocostalis dorsal roots were stimulated.

The animal were fixed in a rigid frame by tapered rods inserted from both sides into the pelvis by a clamp holding spinous processes in the thoracic region. Unilateral fixation of lumbar processes ensured stability of the spine during muscle activation. The skin was sewn onto the frame in a pool which was filled with liquid paraffin. The temperature in the pool was kept at 36–38°C by a red heating lamp. The appropriate ends of the cut nerves or of the roots were stimulated with fine silver-silver chloride wire electrodes. In some experiments, when observing the contractions of the longissimus and iliocostalis muscles, steel needle electrodes insulated to their tips were inserted into the bellies in order to stimulate individual nerve branches supplying the different muscles. Stimuli of 0.3 ms duration were delivered by a Grass S4 stimulator.

Near-isometric muscle contractions were recorded with a Grass FT 03 or FT 10 transducer (bell mass: frequency 500 and 1 000 Hz respectively) connected to the muscles by short lengths (30–60 mm) of material (Mersilene® no. 1). The transducer was connected either to cathode follower and a Grass amplifier or when studying the development of muscle fatigue, to an inkwriter (Grass polygraph). Muscle action potentials were recorded with fine copper wire electrodes insulated except at the tip inserted into the muscles through a hypodermic needle or with DISA needle electrodes. The signals displayed on a Tektronix 50 dual beam oscilloscope and photographed with Grass C4 camera.

The development of muscle fatigue was studied systematically during 10 min periods of stimulation 5 Hz since the further decrease in tension was small in this longer period. The frequency of 5 Hz prevents tetanic contractions thus the blood pumping action of the muscles is preserved and the interference of the blood supply should be minimal (cf. Edström and Lladquist 1973).

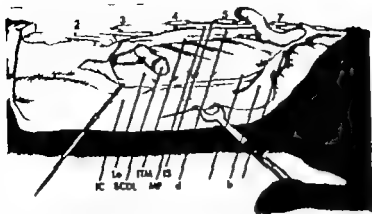
Results

Grass muscular morphology

To be able to analyze the action of the lumbar back muscles exact data on their morphology and the associated connective tissue structures are of primary importance. The available literature seems however to give only generalized descriptions of these muscles in cats and only few data have been found dealing specifically with the muscles in the cat. Large discrepancies were also observed between different descriptions. Hence, a systematic investigation was undertaken to gain more knowledge of the morphology of the muscles in the cat's lumbar back region.

The topographical relations of the muscles under study appear from Fig. 1–4. In Fig. 1 is shown a dorsolateral view of the lumbar region and in Fig. 2 a cross section at the level of the 6th lumbar vertebra. Laterally lie the iliocostalis (IC) and longissimus (Lo) muscles. On the dorsal aspect of the longissimus the sacrocaudalis dorsalis lateralis (SCDL) and intertransversarii mediales muscles (ITM) are located. The medial muscle group consists of the multifidus (MF) and interspinales (IS). The iliocostalis, longissimus, intertransversarii mediales and multifidus muscles are arranged in parallel columns along the lumbar part of the spine while the interspinales are confined to the spaces between the spinous processes (not illustrated in Fig. 2). All these muscles are found in more cranial regions of the trunk as well. The sacrocaudalis dorsalis lateralis extends along the main part of the lumbar trunk and ends at the level of the 2nd lumbar vertebra. Since all the muscles are located dorsolaterally to the vertebral column the muscles execute other movements than flexion and extension.

It is evident that the various groups composing the epaxial muscle system in the lumbar region show great variations in size. The longissimus and iliocostalis constitute almost



1. Dorsolateral area of muscles in the cat's lumbar back region. La, longissimus; IC, sacrocaudal dorsals lateralis (cut and partly freed from Lo); ITM, intertransversarii mediales; IS, intertransversarii laterales; 1, 3 and 7, spinous processes of lumbar vertebrae; a, iliac crest; b and c, covering dorsal muscles, cut and retracted to show structure and fiber directions of underlying ones; d, fascia covering multifidus muscles, cut along dorsolateral aspect of muscles.

the total epaxial muscle mass in the region while the multifidus represent around 10%, sacrocaudal dorsals lateralis about 8% and the intertransversarii mediales together with the interspinales only a few percent.

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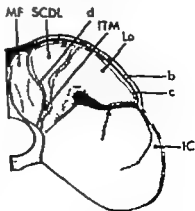


Fig. 2. Cross section at level of 6th lumbar vertebra. Fasciae and muscles marked as in Fig. 1. Darker dotted area of Lo shows localization of muscle tissue appearing red in color. Arrow indicates appropriate position of fusion between lateral and medial connective tissue components of intertransversarii.

avoid mechanical disturbances from other muscles the central ram of the spinal nerves were cut. Experiments applying central root stimulation. For elicitation of reflex contractions of the longissimus iliocostalis dorsal roots were stimulated.

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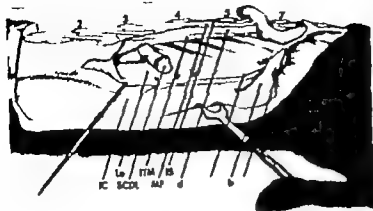
Results

Gross muscular morphology

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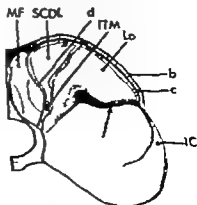


1 Dorsolateral view of muscles in the cat lumbar back region. Lo, longissimus; IC, iliocostalis; L, sacrocaudalis dorsalis lateralis (cut and partly freed from Lo); ITM, intertransversarii medialis; IS, interspinalis; 1, 5 and 7 spinous processes of lumbar vertebrae; a, iliac crest; b and c covering dorsal muscles, cut and retracted to show structure and fiber directions of underlying d, d, fibers covering superficial muscles, cut along dorsolateral aspect of muscles.

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2 Fig. 2. Cross section at level of fifth lumbar vertebra. Fasciae and muscles marked as in Fig. 1. Darker dotted area of Lo shows localization of muscle tissue appearing red in color. Arrow indicates approximate position of fusion between lateral and medial connective tissue components of intermuscular septum.

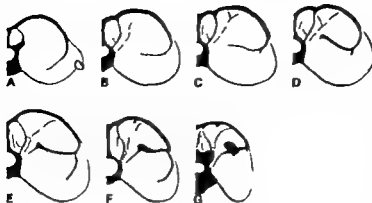


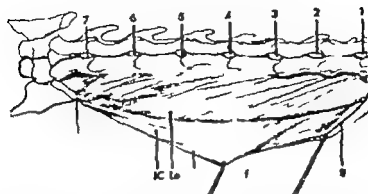
Fig. 3. Cross sections at various levels of lumbar region showing organization of fasciae and muscle fibers as well as outlines of muscle fibers. A-F sections at levels of spinous processes of 1st lumbar vertebra and G at level of articulations between 4th and 5th lumbar vertebrae.

The fibers composing the inner layer run cranio-laterad at the dorso-lateral aspect, turn medially into the muscle mass. Within the muscle tissue there is a fusion between these fibers and others which run in the longitudinal direction. The arrow in Fig. 2 marks the approximate position of this fusion. Thus a strong septum is formed which divides the lateral muscle mass into a ventral and a dorsal part. The septum runs along the back region in an almost horizontal plane and is attached to the iliac crest. This is borne out by the schematic cross sections at various levels of the lumbar region in Fig. 3 A-G. At the level above the 4th lumbar vertebra (A-C) the septum is thin and exclusively made up of fibers entering the muscle from the lateral side. At and below the level of the 4th vertebra the septum is very prominent and located more dorsally than at higher levels (D-G). At the lower levels there is an additional thinner layer of connective tissue medial to the septum. At the most caudal levels this layer reaches the vertebrae (F-G). The ventral part of the muscle mass is subdivided by a septum running in a nearly sagittal plane. This septum is well developed at low levels in the lumbar region (D-F) and appears to be a division of the inner prominent layer covering the dorsal aspect of the muscular system.

Another fascia runs along the lumbar region and separates the medial muscles from the other ones (Fig. 1 d 2 d 3). The fascia is made up of fibers extending in dense bands from the mamillary processes towards the supraspinous ligaments and the spinous processes. Close to the midline the fascia adheres to the covering connective tissue layer (Fig. 2). Within the compartment thus formed there are additional connective tissue structures but their organization has not been studied in detail.

Accordingly the back muscles in the lumbar region are divided into two compartments, i.e. a medial one which contains the multifidus and interspinales and a lateral one containing the iliocostalis, longissimus, intertransversarii mediales and sacrocaudalis dorsalis lateralis. The latter compartment is further subdivided by various septa composing surfaces on which the muscles insert. Hence they have an important function in the mechanics of the muscular system. Their design exhibited minor variations in the cats investigated.

Previous descriptions of the lateral muscles in the lumbar region are somewhat conflicting. The longissimus, as described by Reighard and Jennings (1961) and by Taylor and Weber (1951), fills out the major part of the space between the spinous and the transverse processes. According to the same authors the iliocostalis is a thin layer of muscle lying deep to the longissimus and distinguishable in the uppermost lumbar region.



4 Dorsal view of lumbar region. Structures marked as in Fig. 1. Muscles removed and inner (antral) (a) or directed to show longissimus and its tendinous origins () at accessory processes (f). 1 upper region 2 pale bundles (g) insert into inner dorsolateral surface of iliacus. Illocostalis lies ventral to longissimus and appears indistinctly through fascia.

criptions the illocostalis is connected to the iliacus and the well developed connective tissue septum is regarded as a border between the two muscles in the lumbar region of cats as well as dogs (Bogorodsky 1930, Harrevel and Kok 1934, Rijnberk and Kaiser 1935), so in the present study the septum is considered as a border between the longissimus and illocostalis but it should be emphasized that other interpretations may be justified as well.

The longissimus muscle extends from the lateral aspects of the vertebrae and the accessory corners. The muscle fibers run caudolaterad and insert onto the medial parts of the prominent intermuscular septum and the medial aspect of the iliac bone (Fig. 1 2). Superficial roots of the muscle appear pale in contrast to deep and medial, i.e. central, bundles which are red in color. The position of the red portion of the muscle is indicated in Fig. 2 by a darker dotted area. The portion lies immediately dorsal to the intermuscular septum and can be seen along that part of the lumbar region where the septum is well developed, i.e. from the level of the 4th lumbar vertebra to the iliac crest. At the lower levels of the region the red bundles are turned to the ventral direction also by the thinner septum (cf. Fig. 2 and 3 E, F G). In other directions there are no sharp borders to the pale muscle tissue.

Fig. 4 gives dorsal view of the muscle. The fascial layers are retracted and the muscles coated medially and dorsally have been removed in order to expose the oblique tendon-like formations on the dorsal surface of the longissimus. These structures originate from the accessory processes of the last thoracic and the upper lumbar vertebrae and become progressively thicker as they extend caudolaterad. The areas between the tendons are covered by thin fascia separating the underlying muscle from the medial intertransversarii and the iliac muscle. The figure also demonstrates that muscle fibers originating at thoracic vertebrae insert onto the lateral and dorsal inner surface of the covering layer (in contrast to the insertions of the lumbar segments of the muscle). The longissimus is multisegmentally innervated by intermediate branches of the dorsal ramus. The nerves enter the muscle at the various segmental levels just beneath its dorsal surface.

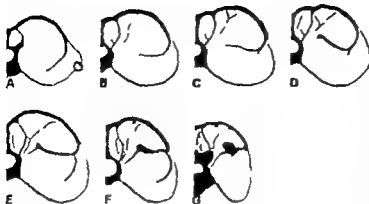


Fig. 3. Cross sections at various levels of lumbar region showing organization of fasciae and muscles as well as outlines of muscles (Fig. 1-2). A-F sections at level of spinous processes of 1st-6th lumbar vertebrae and G at level articulations between 6th and 7th vertebrae.

The fibers composing the inner layer run cranio-laterad at the dorsolateral aspect and turn medially into the muscle mass. Within the muscle tissue there is a fusion between the fibers and others which run in the longitudinal direction. The arrow in Fig. 2 marks the approximate position of this fusion. Thus a strong septum is formed which divides the lateral muscle mass into a ventral and a dorsal part. The septum runs along the lumbar region in an almost horizontal plane and is attached to the iliac crest. This is borne out by the schematic cross sections at various levels of the lumbar region in Fig. 3 A-G. At the level above the 4th lumbar vertebra (A-C) the septum is thin and exclusively made up of fibers entering the muscle from the lateral side. At and below the level of the 4th vertebra the septum is very prominent and located more dorsally than at higher levels (D-G). At the lower levels there is an additional thinner layer of connective tissue medial to the septum. At the most caudal levels this layer reaches the vertebrae (F-G). The ventral part of the muscle mass is subdivided by a septum running in a nearly sagittal plane. This septum is well developed at low levels in the lumbar region (D-F) and appears to be a division of the most prominent layer covering the dorsal aspect of the muscular system.

Another fascia runs along the lumbar region and separates the medial muscles from the other ones (Fig. 1 d 2 d 3). The fascia is made up of fibers extending in dense bands from the mamillary processes towards the supraspinous ligaments and the spinous processes. Close to the midline the fascia adheres to the covering connective tissue layer (Fig. 2). Within the compartment thus formed there are additional connective tissue structures but their organization has not been studied in detail.

Accordingly the back muscles in the lumbar region are divided into two compartments, *viz.* a medial one which contains the multifidus and interspinales and a lateral one containing the iliocostalis, longissimus, intertransversarii mediales and sacrocaudalis dorsalis laterales. The latter compartment is further subdivided by various septa composing surfaces on which the muscles insert. Hence, they have an important function in the mechanics of the muscular system. Their design exhibited minor variations in the cats investigated.

Previous descriptions of the lateral muscles in the lumbar region are somewhat conflicting. The longissimus, as described by Reighard and Jennings (1961) and by Taylor and Weber (1951), fills out the major part of the space between the spinous and the transverse processes. According to the same authors the iliocostalis is a thinner muscle located lateral to the longissimus and distinguishable in the uppermost lumbar region. According to other

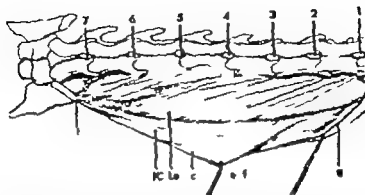


Fig. 4 Dorsal view of lumbar region. Structures marked as in Fig. 1. Muscles removed and sheet fascial layer retracted to show longissimus and its tendinous origins (*f*) at accessory processes (*f*). 1 upper ribon process bundles (*g*) insert into inner dorso-lateral surface of lumbus. Illocostalis lies ventral to longissimus and appears indirectly through lumbus.

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The *iliocostalis* muscle lies ventral and lateral to the longissimus. Caudally the neck is connected to the iliac bone while the cranial portions insert onto the ribs. Superficial bundles are attached to the outer surface of the layer covering the longissimus muscle. The bundles extend cranioventrad to insert into the fascia which limits the muscle in the ventral direction (Fig. 2). Deeper portions of the iliocostalis are connected to the ventral aspect of the muscular septum as well as to the septum running sagittally at the lower levels in the thorax (Fig. 3 E, F). These portions extend towards the transverse processes, the intertransverse ligaments and the lateral aspects of the vertebrae. The nerves supplying the muscle are the lateral divisions of the dorsal rami and are located ventral to the intermediate transverse processes.

The *sacrocaudalis dorsalis lateralis* and the *intertransversarii mediales* muscles are located at the medial dorsal aspect of the longissimus. The tail muscle lies most lateral and as far as possible runs dorsocaudad. The muscle belly extends from the level of the 2nd lumbar vertebra towards the tail. In the lumbar region this muscle inserts onto a narrow strip along the dorsal surface of the longissimus (Fig. 1 2). In lower lumbar sections of the muscle there is a dorsally located tendon which runs caudad (Fig. 3 F G). In the sacral region it gives off a pair of small tendons inserting onto sacral spinous processes. The nerve supply to the tail muscle differs from that to the other back muscles studied. Branches of the sacral spinal nerves collect to a common nerve trunk passing between the sacrocaudalis dorsalis lateralis and the longissimus muscles. The nerve ascends into the lumbar region, taking a long curvilinear muscular course.

The observations made in the present study show that the tail muscle, which some authors have described as the medial division of the longissimus (Reighard and Jennings 1961 Taylor and Weber 1951) is actually a discrete muscle belly connected to the caudal skeleton. Stimulation of the motor nerve results in tail movements. The muscle seems to be analogous to the sacrocaudalis dorsalis lateralis muscle in the dog and it should thus be justified to use the same designation in the case of the cat (cf. Schumacher 1910).

Medial to the tail muscle lie the short bundles of the *intertransversarii mediales* muscle (Fig. 1 2). These fibers connect the mamillary and the accessory processes by insertions into the tendons at the dorsal aspect of the longissimus. The bundles also take origin from the fascia on the lateral aspect of the multifidi. At low levels in the region the bundles are less prominent and pass over fewer intervertebral joints than at the upper levels. Fibers originating from the mamillary process of the 6th lumbar vertebra insert onto tendons connected to the 5th and 4th vertebrae. Hence not more than one vertebra is bridged. Muscle portions located in the upper region sometimes span over three vertebrae; thus, e.g. fibers connecting to the mamillary process of the 4th lumbar vertebra reach the tendon attached to the 1st thoracic vertebra. In the uppermost lumbar region the bundles insert also onto the dorsal aspect of the longissimus not covered by tendons. The medial *intertransversarii* bundles are innervated by medial branches of the dorsal rami entering the muscle from its deep aspect.

The *multifidi* and the *interspinales* muscles are located side by side in the medial compartment of the region. The short and oblique multifidi bundles are interwoven with each other and form an almost homogeneous column along the lumbar spine. The muscle fibers are attached to the mamillary processes and extend cranio-medially inserting onto the adjacent lumbar vertebrae by a complex of connective tissue structures.

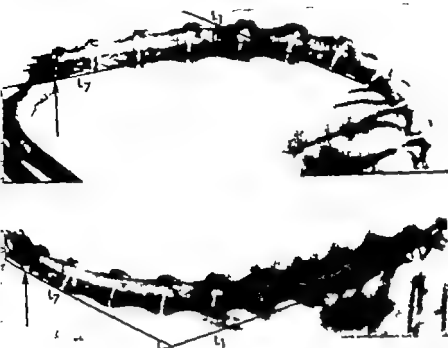


Fig. 5. X-ray pictures of lower spine and pelvic region in lateral view. Flexed (A) and extended state (B). Arrows represent longitudinal axes of sacral bones (S), seventh (L₇) and first lumbar vertebrae (L₁). Indicated are angles between L₇ and L₁, and between S and L₁. Sacrospinous joint at arrow. P, pelvic bone. In the large mobility in the lumbosacral joint as compared with that of lumbar intervertebral centres.

Between the spinous processes the interspinous muscles extend cranioventrad. In addition to the insertions on the spinous processes the muscle bundles also attach to connective tissue structures which run sagittally in the compartment. The interspinous muscles could be identified at the different levels in the lumbar region but in some cats they were weakly developed between the two last lumbar vertebrae. The multifidus and the interspinous muscles are innervated also by medial branches of the dorsal rami. The nerves pass lateral to the superior tubular processes and enter the muscle tissue from the ventral aspect.

As far as the muscles in the medial compartment are concerned, the present observations accord with previous descriptions given e.g. by Kruger (1927) and by Reingard and Jennings (1961) (cf. also Slipper 1946). Thus, it is not possible to distinguish single muscle bundles or different layers of bundles in the lumbar region.

Mobility of the lower spine

In view of the morphological observations made on the longissimus and iliocostalis muscles as well as on the associated connective tissue structures it seemed relevant to study the mobility of the lumbar region, especially in the sagittal plane.

Fig. 5 shows X-ray pictures of the lower spine in a lateral view when flexed (A) and extended (B). By measuring the angles between the longitudinal axes of different vertebrae in

the flexed and in the extended position the mobility of different joints can be estimated. The figure solid lines indicate the axes of the sacral bone (S), the seventh (L₇) and the first lumbar vertebra (L₁). In flexion (A) the axes of the sacral bone and the seventh lumbar vertebra are practically parallel whereas the angle between the last and the first lumbar vertebra is about 30°. In extension (B) the angle between the sacral bone and the last lumbar vertebra is 25° and between the last and first lumbar vertebra 45°. The mobility of the sacral joint (arrow) is thus 25° and between all lumbar vertebrae (L₁-L₇, 6 levels) about 150°. Hence, the lumbosacral joint alone accounts for a large part of the overall mobility in the lower spine and contributes significantly to the marked rotation of the pelvic bone. In the lower thoracic region the mobility of each joint is much more pronounced than in the lumbar region.

Contraction properties and observations on the action of some lumbar back muscles

The medial muscles multifidi and interspinales and the lateral longissimus and iliocostalis are supplied by multiple motor nerves entering each of the muscles at different levels in the lumbar region. In order to find out more about this innervation pattern an initial series of experiments was performed in which the muscles were activated by stimulation of ventral roots or of the corresponding dorsal rami of the spinal nerves at different segmental levels. Stimulation of ventral roots from different spinal segments proved to evoke contractions in different regions along the muscle bellies. Upper lumbar segments supply upper lumbar regions of muscles while lower segments innervate more caudal regions. The particular region of a muscle supplied by a particular segment could most readily be discerned by stimulation at a high frequency causing a tetanic contraction of the muscle fibers. Myotomes of these muscles thus seem to be arranged longitudinally with a relatively small overlap between the adjacent segments. Stimulation of the dorsal rami at corresponding levels along the lumbar spine gave the same pattern. The findings indicate that the motor innervation of the muscles is closely correlated with the organization of the myotomes and exclude the existence of extensive nerve plexuses formed by the dorsal rami.

In these experiments the number of intervertebral joints directly affected by contraction of muscles innervated by one segment was also determined. Stimulating the medial branch of the dorsal ramus, which supplies multifidi and interspinales muscles, yields tension in tendon structures which run more or less sagittally in the medial muscle compartment: they insert onto spinous processes (cf. Fig. 1, 2, 3). Maximum tension is recorded in the tendon which insert on the spinous process corresponding to the segmental nerve stimulated and in the two adjacent spinous processes. Thus, the findings demonstrate that medial muscles when activated by a given segment may act over intervertebral joints at least at three different levels.

Activating the longissimus and iliocostalis by stimulation of ventral roots at different levels results in a tension transmitted to large sections of the lumbar spine and to the pelvic bone. To establish possible differences in action between these muscles, they were selectively activated by needle electrodes and the tension development at the iliac crest as well as along the deep fascial layer was recorded (cf. Methods). Activation of the longissimus in its middle and lower lumbar sections proved to result in maximal tension at the iliac crest while the

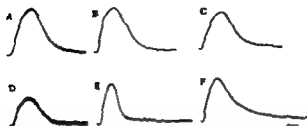


Fig. 6. Isometric twitch contractions of medial muscles multifidus and interspinales (A-C) in response to supramaximal stimulation of their motor nerves arising from L_1 (A), L_4 (B) and L_6 (C) segment and of lateral muscles longissimus and iliocostalis (D-F) evoked by supramaximal stimulation of ventral roots D_1 , L_4 (E) and L_6 (F). Contraction times in A 42 ms, B 36 ms, C 39 ms, D 35 ms, E 36 ms and in F 37 ms. Time bar 20 ms.

tion along the fascial layer was lower. When stimulating the upper lumbar section of the spine more tension develops along the fascial layer than at the iliac crest. Contractions of the iliocostalis yield forces that are transmitted along the inner fascial layer and to the iliac bone. Hence, the middle and lower parts of the longissimus seem to primarily act on the pelvic girdle. Other portions of the muscle operate on the lumbar spine as does the iliocostalis which in addition is connected to the pelvic girdle.

In view of the observations presented in the previous sections and the organization of medial myotomes an investigation of the contraction properties at different levels along the lumbar region was performed.

In Fig. 6 A-C are shown isometric twitch contractions at optimal muscle length of the medial muscles multifidus and interspinales in response to supramaximal stimulation of their motor nerves arising from the 1st (A), 4th (B) and 6th (C) lumbar segment (*cf.* Methods). In these recordings the contraction times (from onset to peak) vary between 36 and 42 ms.

The recordings in Fig. 6 D-F were made under conditions comparable to those in A-C and show isometric twitches in the lateral muscles longissimus and iliocostalis evoked by stimulation of ventral roots originating from the 1st (D), 4th (E) and 6th (F) lumbar segment. In these recordings the contraction times vary between 36 and 35 ms. The twitch responses in E and F show a longer relaxation phase than that in D. This phenomenon will be considered below.

Thus, it is evident that different regions of the medial muscles have about equal contraction times, as have also different regions of the lateral ones. However, the former muscles generally contract somewhat more slowly. This is borne out by Table 1 summarizing ex-

TABLE 1. Contraction times of cat lumbar back muscles.

Muscle	Segment	Range of contraction times (ms)	Mean contraction times (ms)	Number of experiments
Multifidus, interspinales	L_1 , 4	20-42	34	10
Longissimus, iliocostalis	L_4 -6	24-35	29	15

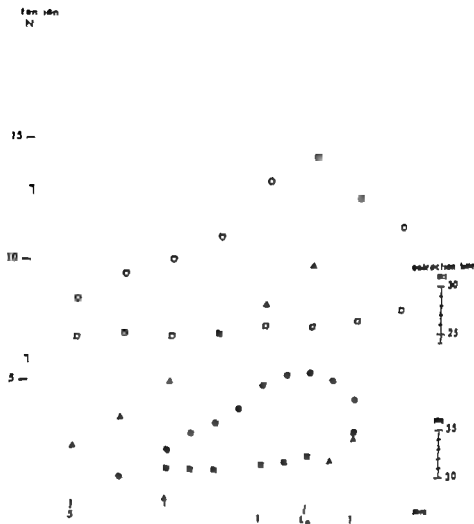


Fig. 7 Isometric twitch tension (circles), passive tension (triangles) and contraction time (rectangles) of medial muscles multifidus and interspinales (filled symbols) and of lateral muscles longissimus and iliocostalis (open symbols) plotted against muscle length. On the vertical line 10 mm marks muscle length giving 50% twitch amplitude.

experimental data on contraction times of the muscles (values obtained on stimulation of different segments have been pooled and mean values calculated)

As in the case of *c.g.* muscles in the hind limb the contraction times of the medial and lateral muscles increase with lengthening of the muscles. The medial muscles exhibit the most prominent changes. This is illustrated in Fig. 7 in which the contraction times (rectangles) of the multifidus and interspinales (filled symbols) and of the longissimus and iliocostalis (open symbols) are plotted against muscle length. The twitch tension (circles) and the passive tension (triangles) developed in the muscles are plotted in a similar way. The maximal twitch tension in the lateral muscles is 2–3 times higher than in the medial ones. The former muscles are also capable to attain higher tension within a wider range of muscle lengths which indicates a difference in the length of the muscle bundles. Thus, e.g. at 4 mm the lateral muscles develop about 60% of their maximal tension but the medial muscles only about 20%. The passive tension recorded during lengthening of the muscles



Fig. 8. Superimposed recordings of isometric twitch contractions of medial muscles multifidi and interspinales (A) and of lateral muscles longissimus and iliocostalis (B) in response to stimulation of different sites of the motor nerve arising from the L₄ segment and the ventral root of the L₄ segment respectively between 20 ms.

in both cases and this distinguishes them from the hind limb muscles where the force is very low up to lengths resulting in maximal active tension (cf. e.g. Butler Eccles & Eccles 1960).

Muscle action potentials elicited by supramaximal nerve stimulation were of about the same duration in the medial as in the lateral muscles. The potential duration in the multifidi and interspinales ranged from 4.2 ms to 8.0 ms (mean 5.2 ms) and in the longissimus and iliocostalis from 4.3 ms to 5.0 ms (mean 4.7 ms).

Heterogeneity of motor unit populations

Contraction times of motor units are usually related to the diameter of their motor nerve axons; thus fast units are innervated by coarser fibers than are slow units (see e.g. Wuerker, Chedron and Henneman 1965). Since the threshold for electrical stimulation differs for the fibers of different sizes it is sometimes possible to distinguish between motor units with different contraction times by applying motor nerve stimulation of different intensities (e.g. Andersen and Sears 1964).

Fig. 8 shows superimposed recordings from experiments utilizing this technique in which twitch contractions of the medial (A) and of the lateral muscles (B) have been elicited by stimulation of their nerves originating from the 4th and 5th lumbar segment respectively. In contractions elicited by low-strength stimulation in the former muscles attain peak tension somewhat more quickly than those elicited by activation of high-threshold fibers, thus indicating a mixture of motor units with different contraction times. The situation is essentially the same in the longissimus and iliocostalis muscles but in addition there is a late step in response to summation of medium and high-threshold fibers originating from the 4th lumbar segment (cf. also Fig. 6 E, F). This indicates the presence also of a slowly contracting population of muscle fibers in middle and lower regions of the lateral muscles which

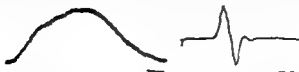


Fig. 9. Reflex action potential (right) and isometric contraction (left) elicited in lateral muscles by electrical stimulation of the ipsilateral 4th lumbar dorsal root. Electrical response recorded from central region of longissimus at level of 5th-6th vertebrae. Time bars 2 ms (right), 20 ms (left).

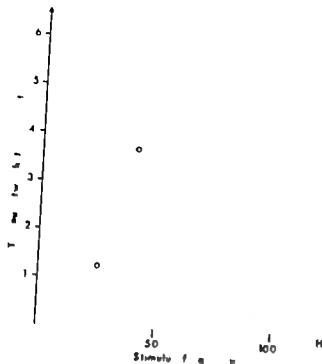


Fig. 10. Relation between stimulus frequency and tension developed by medial muscles multifidus and interspinal (triangles) and the lateral muscles latissimus and iliocostalis (circles).

are supplied by lower lumbar segments. A heterogeneity of this kind is found in other muscles of the cat as well (Denny Brown 1979, Gordon and Phillips 1953).

Further evidence for a slow component was obtained in experiments with reflex activation of the lateral muscles. Fig. 9 shows mechanical (left) and electrical (right) recordings for the muscles in response to low-strength stimulation of the 4th lumbar dorsal root. The time to peak tension is about 100 ms and corresponds well to the late hump in the mechanical response to ventral root stimulation (Fig. 8 B). The action potential is recorded from the central part of the longissimus muscle, located dorsally to the intermuscular septum. Compared with recordings from other parts of the muscles, the maximum amplitude of the electrical response was always recorded in this particular region of the longissimus.

The duration of the muscle action potential is 4.5 ms which is in the same range as the duration of the electrical response following ventral root stimulation. This implies that the time to peak tension cannot be attributed to repetitive discharges associated with the reflex activation of the muscle but represents the contraction time of slow twitch units located in the longissimus. The nature of this reflex will be further dealt with in another paper (Carlson 1978 b).

Responses to repetitive stimulation

The mechanical responses to repetitive stimulation of muscle portions innervated from different segments were studied in a series of experiments, and in Fig. 10 typical results are given. The figure shows the relation between stimulus frequency and tension developed, and it appears that summation of mechanical responses in the medial muscles (triangles) starts at 10–15 Hz and is total at about 50 Hz (apparent fusion of Bullock and Lewis 1960) whereas the corresponding values for the lateral muscles (circles) are 15–20 Hz and show



11 Time course of twitches of multifidus and interspinales (A) and of longissimus and iliocostalis (B) and by supraspinal stimulation of their nerves from L_4 and L_5 segments respectively. Markings: pointing upwards indicates stimulation at 5 Hz, downwards at 0.5 Hz.

Hz. Somewhat smaller twitches started at a somewhat lower frequency in the middle and inner regions of the two lateral muscles. This is likely to be due to the slowly contracting myotome located in these regions.

Tetanus/twitch tension ratios were determined in 11 experiments on the two muscle arms and proved generally to be higher for the lateral than for the medial muscles. The ratios for the former muscles ranged from 4.8 to 7.5 (mean 5.9) and for the medial muscles from 2.8 to 5.2 (mean 3.5).

Performance of lumbar back muscles during prolonged activity

In a total of 13 experiments the medial and lateral muscles were studied with respect to their ability to maintain contractile tension during prolonged activity. Fig. 11 illustrates the time course of twitch tension in the multifidus and interspinales (A) and in the longissimus and iliocostalis (B) during a period of motor nerve stimulation at 5 Hz. The recordings start when stable twitches are obtained at 0.5 Hz. At the marking pointing upwards the stimulus frequency is instantly increased to 5 Hz. Following an initial atreous effect which is most evident in the longissimus and iliocostalis, the twitch amplitudes decrease considerably during 10 min of stimulation at 5 Hz. At the end of this period the medial muscles retain 40% of their initial tension while the corresponding value for the lateral muscles is 21%. The marking pointing downwards indicates a change of the stimulus frequency back to 0.5 Hz. Twitch contractions showing slight post-tetanic potentiation are then recorded in both muscle systems before a final twitch amplitude is reached which for the medial muscles is 24% of the initial tension and for the lateral muscles 22%. Recordings of compound muscle action potentials at different times after the onset of stimulation showed that the potentials from the multifidus and interspinales as well as from the longissimus and iliocostalis declined markedly during stimulation at 5 Hz. Thus, also other factors than a failure of the contractile mechanism seem to be responsible for the decrease of the contractile tension.

In most experiments involving muscle portions innervated by different segments, the medial muscles retained a higher proportion of the initial twitch tension than the lateral muscles during the later part of the period of stimulation at 5 Hz. During the subsequent

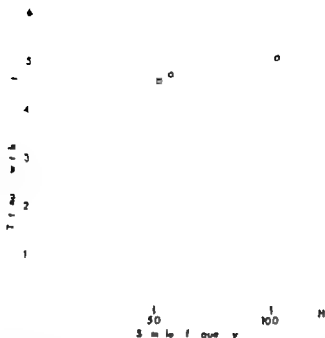


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and iliocostalis on the one hand and the pelvic girdle on the other hand is apparently of great importance for the execution of movements and for the maintenance of stability in all quarters during different types of locomotion. In the uppermost lumbar region erector is weakly developed and the mobility is greater. Thus, it seems likely that the erectors in this region operate more exclusively on the axial skeleton than do the lower parts of the muscles.

A study of the contractions in the longissimus also suggested a functional difference between the various sections of the muscle. Lower sections are probably more effective in control of the posture in the lumbosacral joint than are upper parts of the muscle. Since intervertebral joint is more mobile than other joints in the lumbar spine the longissimus has to be capable of large variations in length. Actually this is also the case, due to the specific morphology of the muscle. In middle and lower sections of the lumbar region the medial aspect of the muscle is smooth, with no insertions onto the covering layer and may slide beneath the connective tissue sheet. In this context it is of relevance to state that the slow component of the longissimus is able to extend the lumbosacral joint and might therefore be expected to take part in the tonic control of the position of the pelvic girdle vis-à-vis the lumbar spine (cf. Carlson 1978 b).

The medial division of the back muscles constitutes a minor portion and is formed by small bundles connecting adjacent vertebrae. Contractions elicited by stimulation of a motor nerve at a given level affect only restricted sections of the lumbar spine. Hence, these bundles are primarily involved in the control of movements and posture of one vertebra relative to other whereas the longissimus and iliocostalis act on larger sections of the lumbar spine. As medially located muscles do not exhibit any distinct separation into different layers, it might well correspond to the limited mobility in the lumbar region since in other regions, where the spine is more supple, the differentiation of the muscles into separate layers is more pronounced.

innervation

The lumbar back muscles are supplied by the dorsal rami of the lumbar spinal nerves. The lateral and intermediate branches of these rami innervate the iliocostalis and longissimus respectively and the medial branches the intertransversarii mediales, multifidus and interpharyngeales. The sacrocaudalis dorsalis lateralis is supplied by a separate nerve trunk, ascending from the sacral region. In previous descriptions (Reighard and Jennings 1961, Taylor and Weber 1951) only two branches have been identified, viz. a medial and a lateral one. The existence of three separate branches of the dorsal rami has recently been observed also by Jørgensen (1976).

In their investigations of the motor supply of the dog back muscles Harnsveld and Kok (1932) and Rijnberk and Kabaer (1935) were able to demonstrate that specific segmental motor fibers located in a particular region along the muscle bellies. The myotomes composing the muscle were found to be arranged after one another in a row. In the present experiments on the cat a similar pattern was observed. Furthermore, stimulation of peripheral nerves entering the muscles at different levels along the spine was shown to evoke contractions in that part of the muscle which is activated by stimulation of the corresponding

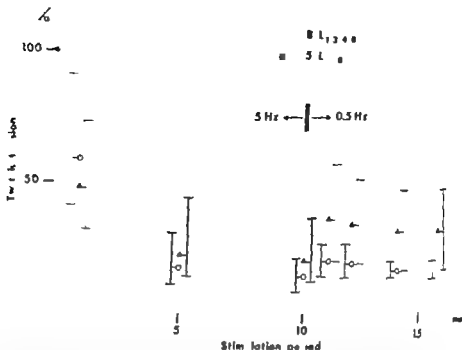


Fig. 1. Mean isometric twitch tension developed by multifidus and interspinales (triangles) and by iliocostalis and iliocostalis (circles) on continuous supramaximal nerve stimulation 5 Hz for 10 min followed 0.5 Hz for 6 min. Bars indicate range of values. Segments investigated and number of experiments per figure.

period the difference between the muscles was more pronounced ($p < 0.001$ at 16 s). Segmental variations in the susceptibility to fatigue may be present but this question has not been studied specifically. The experimental findings during prolonged muscular activity have been summarized in Fig. 12.

Discussion

Gross muscular morphology

Great mobility of the axial skeleton is a characteristic feature of a cursorial mammal as the cat. The spine is flexed and extended during fast locomotion and hence such animals are called dorsomobile runners (Gambaryan 1974 *cf.* Hildebrand 1959). During slow types of locomotion movements of the axial skeleton are less obvious (Goslow, Reel and Stuart 1973). However, the mobility in the vertical plane differs in various joints of the axial skeleton as shown in the present study (Fig. 5). Each lumbar joint has less mobility than the lumbosacral joint and the joints in the lower thoracic region which are the most mobile (*cf.* Auer 1914; Slijper 1946).

The mobility of the axial skeleton is reflected in the morphology of the muscles and organization of the associated connective tissue structures. The lateral muscles longissimus and iliocostalis are connected to the spinous processes by the inner fascial layer in the thoracic region (Fig. 1, 2). This is essential for their action as extensors of the spine. The two muscles are also connected to the pelvic girdle by the intermuscular septum which is most prominent at the lower levels of the lumbar region. The strong septum permits the force developed by the muscles to be directly transmitted to the pelvic bone. The association between the

lower back muscles is fast contracting and shows a marked fatigability on prolonged stimulation and that only a small fraction of the muscle mass in the region may have tonic functions. This is of significance when considering their role in motor events.

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ventral roots. As indicated in Results, this implies that the multiple innervation is connected to the organization of the myotomes. However, the existence of nerve plexuses cannot be ruled out and this possibility should be further investigated.

This particular innervation pattern is similar to that of axial muscles in fishes and differs from the innervation of extremity muscles in mammals. In fishes myosepta separate myotomes from each other. Such structures have not been found in the cat's dorsal muscle. Hence, the segmental organization of the myotomes should not be overemphasized. Nevertheless, it is likely that different sections along a muscle are reflexively operated by different spinal segments. Local differences in the activity of one and the same back muscle have been observed on application of nociceptive stimulation to back skin (Carlson and Lissander 1976). Such differences can also be observed in the dog's back muscles during locomotion (Tokuriki 1973, 1974). Observations of this kind indicate that the multiple innervation of the back muscles has direct consequences for the design and interpretation of experiments dealing with reflex patterns as well as for studies of the activity of the muscles during locomotion.

Mechanical properties of lumbar back muscles

Since the total mobility of the spine is equal to the sum of the mobility of all the joints, it might be expected that muscle bundles connecting adjacent vertebrae, such as the *medialis* and *interspinales*, should be able to develop high tension only within a narrow range of length in contrast to muscles controlling movements and posture over larger sections of the spine. The experimental results lend support to this hypothesis since the *medialis* could be shown to be more decisive for the twitch amplitude of the medial muscles than the *longissimus* and *iliocostalis*. This is probably due to differences in the organization of the whole muscles and should not be ascribed to differences in the properties of the individual muscle fibers.

The contraction times recorded for the lumbar back muscles are within about the same range as those observed for some of the fast extremity muscles like *gastrocnemius*, *flexor hallucis longus* and *flexor digitorum longus* (Wills 1942, Bulter, Eccles and Eccles 1966). However, the lateral muscles *longissimus* and *iliocostalis* contract slightly faster and are generally more susceptible to fatigue than the medial *multifidi* and *interspinales*. Furthermore, the time course of the twitches in the latter muscles did not vary much in different regions along the lumbar spine, in contrast to the mechanical responses of the lateral muscles *longissimus* and *iliocostalis*. Activating the latter muscles by ventral root stimulation of lower lumbar segments (L_4-L_6) suggested the presence of a slowly contracting portion. Root activation indicated that slow twitch fibers are located in the central region of the *longissimus* muscle along the middle and lower lumbar spine. The differences in contractile properties between the lateral and the medial muscles are in accordance with recent findings on the histochemical fiber composition of the various lumbar back muscles (Carlson 1978a). Of course viscous and elastic elements may contribute to differences in contractile properties between the various muscles as well as between different regions of a single muscle belly but these factors have not been analyzed specifically.

Perhaps the most striking observations in the present study are that the main part of

4-5.9 as is also the fusion frequency 70 Hz, and the fatigability of these muscles is pronounced. Another difference is that a portion of the lateral muscles, located in the r region of the longissimus, contracts more slowly than upper parts. No such segmental zones are found in the medial muscles.

The present work was undertaken to study the muscle fibers in the cat's lumbar back muscles by histochemical methods in order to find out how the fiber composition of the back muscles is related to their contraction properties. Thus biopsies from different parts along the muscles and at various depths were analyzed to determine the presence and distribution of different types of muscle fibers. The analyses reveal that the muscles are composed of three main fiber types and that their distribution varies markedly in the different muscles.

A preliminary account of some of this material has been given elsewhere (Carlson 1976).

Material and methods

Histochemical analysis. Biopsy material from different levels and regions of the longissimus, iliocostalis, transversus medialis, multifidus, interspinales and the sacrocaudalis dorsalis lateralis (see Table 1 for nomenclature as in Carlson 1976) was obtained from eight cats (3.2-4 kg) anesthetized by *i.p.* injection of Nembutal® (30-40 mg/kg b.w.). The biopsy material was immediately frozen in isopentane previously cooled with liquid nitrogen. Sections 10-15 µm thick were cut on a cryostat at -20°C. Staining methods: myofibrillar adenosine triphosphatase (ATPase; Padykula and Herman 1953) following preincubation at pH 9.4, 4.6 and 4.3 (Brooke and Kaiser 1969, 1970), NADH-tetrazolium reductase (NADH-t; Scarpa, Hess and Pearce 1958), succinic dehydrogenase (SDH; Nachlas *et al.* 1957), phosphorylase (Schmidt and Karni 1953 as modified by Erlank and Palmak 1961), lipids (Carlson and Drury 1957) and glycogen (PAS technique as described by Dubowitz and Brooke 1973). Some slight modifications are made (see Nyström 1968). For further details see Pearce (1960).

General histochemical characteristics of muscle fibers

The histochemical characteristics of individual fibers were studied by photographic registration and comparison of consecutive cross sections stained for the different enzymes and substrates.

Myofibrillar ATPase. In the sections preincubated at pH 9.4 two distinct fiber types can be identified in all the six muscles studied. The fibers that have low activity of myofibrillar ATPase, type I in the nomenclature by Brooke and Kaiser (1970), stain light and the fibers with high activity type II according to the same nomenclature, appear dark in the sections from the longissimus (Fig. 1 A and 2 A). The medial intertransversarii (Fig. 3 A and 4 A), the multifidus (Fig. 5 A) and the interspinales (Fig. 6 A).

The staining pattern of the different fiber types may be altered by varying the conditions of preincubation (see e.g. Brooke and Kaiser 1970). Following preincubation at pH 4.3 the ATPase reaction is inhibited in type II fibers while no inhibition occurs in type I fibers. Hence, the staining pattern is reversed, type II fibers stain light and type I fibers dark. This reversal is evident when comparing the serial cross sections from the medial intertransversarii preincubated at pH 9.4 (Fig. 4 A) and at pH 4.3 (Fig. 4 B). However, section type II fibers are not completely inhibited and stain moderately at pH 4.3. These conform to the type II C fibers in the classification by Brooke and Kaiser (1970) and in the figures each fiber is indicated by cross in general, very small number of type II C fibers were identified in the lumbar back muscles investigated and their relative proportions are therefore not considered.

Preincubation at pH 4.6 results in a markedly inhibited ATPase reaction in some type II fibers but not in others. This modification of the ATPase reaction may therefore also be utilized to demonstrate further subdivisions among the type II fibers. This is illustrated in Fig. 1 and 3 showing consecutive cross sections from the longissimus and the multifidus muscles respectively. In Fig. 1 two type II fibers, indicated by an 'X' stain dark for myofibrillar ATPase after preincubation at pH 9.4 (A) whereas only the lowermost fiber is inhibited and appears light in the section preincubated at pH 4.6 (B). Such fibers are termed type II A while fibers with intermediate stainability are designated type II B in the nomenclature applied.

Histochemical fiber composition of lumbar back muscles In the cat

By

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Abstract

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A histochemical analysis has been performed of the activity of myofibrillar ATPase, NADH-dependent reductase, succinic dehydrogenase and phosphorylase and of the content of fat and glycogen in the muscles of the cat lumbar back region. The correlation between the fiber composition and the previously studied contraction properties of the muscles was analyzed. All muscles contain fibers with a low activity (type I) and such with a high activity (type II) of myofibrillar ATPase following preincubation at pH 9.4. Type I fibers showed either a low (type II A) or an intermediate (type II B) reaction when stained for ATP preincubation at pH 4.6. Type I fibers have a high, II A an intermediate-high and II B fibers a low level of oxidative enzymes. The longissimus, iliocostalis and sacrocaudalis dorsalis lateralis muscles are characterized by high percentages of type II B fibers and low proportions of type I and type II A fibers. The central region of the longissimus which is connected to a well developed intermuscular septum is composed of high proportion of type I fibers. The multifidus interspinales and intertransversarii muscles have higher proportions of type I and type II A fibers than the other muscles in the region.

The morphology and contraction properties of the lumbar back muscles in the cat were described in a previous paper (Carlson 1978). Medially located muscles such as the multifidus and interspinales are made up of bundles taking a shorter and more oblique course than the lateral longissimus and iliocostalis. Hence the medial muscles connect a smaller number of vertebrae than the two lateral muscles which, in addition, are strongly connected to the pelvic girdle. The back muscles have a multisegmental innervation; the different spinal segments supply different regions along a muscle belly in such a way that the myotomes composing a muscle are arranged after one another in a row.

The lumbar back muscles have short contraction times and fatigue fast on prolonged stimulation. There are however some differences between the medial and lateral muscles. Thus the medial muscles as represented by the multifidus and interspinales have a mean contraction time of 34 ms, a tetanus/twitch ratio of 3.5 and an apparent fusion frequency of 50 Hz. In the longissimus and iliocostalis which constitute the major portion of the lateral muscles the mean contraction time is somewhat shorter, 29 ms, the tetanus/twitch ratio

§ Biopsy material from lumbar back muscles.

	Vertebral levels	Number of specimens	Cat
muscle			
vertical	L ₄₋₅	16	1-8
p	L ₄₋₅	3	3-5
p and medial; "central"	L ₄₋₅	7	3-8
muscle			
vertical	L ₄₋₅	12	1-8
p	L ₄		6, 8
transversal muscles			
vertical	L ₄₋₅	11	1, 3, 8
p			
vertical	L ₄	11	3-5, 7, 8
p			
vertical	Th ₁₂ -L ₁	7	4, 5, 7, 8
p			
vertical	not specified	4	2, 5

1 C and 2 B represent sections stained for NADH₂-TR and demonstrate other differences between the two parts of the muscle. I the central region (Fig. 2 B) the type I fibers appear slightly lighter than they do in the superficial region. In the latter part of the section (Fig. 1 C) the type II fibers exhibit varying staining intensities for the oxidative type while such gradations are less obvious III the type II fiber population of the central part of the muscle. Neither did specimens obtained from the latter region stained for ATPase, incubation pH 4.6, display any distinct differences between various type II fibers. Thus, a subdivision into subdivisions of type II fibers III the central part of the muscle becomes uncertain. In the peripheral part of the muscle, type II A fibers constitute about 14% and type II B fibers 77% while the central portion is made up of about 31% of type II fibers. In the latter region type II C fibers are more numerous than in any other of the lumbar back muscles investigated. When comparing the sections from the two regions of the muscle it is also evident that the diameters of the fiber populations differ. In superficial portions type I fibers are small (38 μ m) and the type II fibers considerably larger (mean value of

TABLE II Histochemical staining reactions of lumbar back muscle fibers.

Staining	Type I	Type II A	Type II B
myoglobin			
ATPase preincubation at			
pH 9.4	low	high	high
pH 4.6	high	low	intermediate
pH 4.3	high	low	low
NADH ₂ -TR	high	intermediate-high	low
CDH	high	intermediate-high	low
AT	high	intermediate-high	low
phosphorylase	low	high	high
glycogen	low	high	high

Oxidative enzymes and fat In Fig. 3 a type I fiber from the multifidus, lightly stained for myofibrillar ATPase preincubation at pH 9.4 (A) and darkly stained for ATPase preincubation at pH 4.6 (B) indicated by an arrow also in serial cross sections stained for NADH₂-TR (C), SDH (D) and fat (E). The fiber is well supplied with the two oxidative enzymes and fat. Type I fibers in the lumbar back muscles exhibiting an intermediate stainability for the two oxidative enzymes and fat were only occasionally identified. In the same figure, two type II fibers darkly stained in A are indicated by an arrow. The type II A fiber has an intermediate, and the type II B fiber reacting intermediate in B, a low activity of the oxidative enzymes and a low content of fat (C, D, E). Type II A fibers with a high activity for the oxidative enzymes and a high content of fat are occasionally found in the muscles. Thus, in general type I fibers in the lumbar back muscles have a higher stainability for the oxidative enzymes and fat than the type II A fibers (Fig. 1, 3). Type II C fibers show a staining pattern for oxidative enzymes and fat similar to that of the type II A fibers (Fig. 4). It is typical of the individual fiber that the reactions for NADH₂-TR, SDH and fat are parallel. This is in agreement with the staining patterns of extremity muscles in mammals. It is noted that a separation of the type II fibers into two subgroups (corresponding to II A and II B) can be made on the basis of the staining pattern in the reaction for NADH₂-TR as well as on the pattern observed in sections stained for ATPase preincubation at pH 4.6. The former method was sometimes hindered by such a separation since it was more often technically successful than the ATPase procedure.

Phosphorylase and glycogen In Fig. 1 the staining reactions for myofibrillar ATPase, preincubation at pH 9.4 (A) and at pH 4.6 (B), NADH₂-TR (C), phosphorylase (D) and glycogen (E) are compared in serial cross sections from the longissimus. An arrow marks the position of a type I fiber which is weakly for ATPase, pH 9.4, phosphorylase and glycogen but gives an intense reaction for ATPase, pH 4.6 and NADH₂-TR. In the same figure two type II fibers are indicated by an arrow. These fibers exhibit high activity for myofibrillar ATPase, pH 9.4 and phosphorylase as well as a high content of glycogen while the stainability of the fibers for ATPase, pH 4.6, and NADH₂-TR differs. Hence, the phosphorylase activity and the glycogen content are largely parallel with the activity of myofibrillar ATPase and preincubation at pH 9.4 (i.e. low in type I and high in type II fibers).

The various types of fibers found in the six lumbar back muscles are summarized in Table II. The results obtained show that the muscle fibers may be divided into three main types exhibiting staining patterns similar to those of fibers in hind limb muscles of the cat (Burk *et al.* 1973; for review see e.g. Close 1975). In general the type I fibers show a high stainability for the oxidative enzymes while the type II A fibers exhibit intermediate and the type II B fibers low reactions.

The relative proportions of different types of fibers in a specimen were calculated on examination of about 1000 fibers except in specimens obtained from the interspinous and the lower parts of the lumbar transversarii mediales muscles in which about 300 fibers were examined.

The size of the muscle fibers was determined in specimens from one cat by estimating the fiber diameter (Dubowitz and Brooke 1973) in cross sections stained for NADH₂-TR. Approximately 200–400 fibers were measured in randomly selected areas of the cross sections following identification in consecutive sections stained for ATPase. Standard deviation of the mean fiber diameter was adjusted (Sheppard's correction, see Bailey 1969).

Results

Fiber composition of individual muscles

In total, 73 specimens obtained from various parts of the six muscles were examined (see Table I). The general histochemical characteristics of the muscle fibers are described above and summarized in Table II. The distribution as well as the diameters of the various fiber types in certain regions of the lumbar back muscles are summarized in Table III.

The longissimus Fig. 1 A shows a cross section from a superficial muscle portion at the level of the 5th lumbar vertebra stained for myofibrillar ATPase, pH 9.4. Most fibers (91%) are darkly stained and thus of type II. In Fig. 2 A is shown a central portion at the same level of the muscle stained for ATPase, pH 9.4. Macroscopically this part of the muscle appears red in color and is made up of a high proportion of type I fibers (69%). These fibers stain somewhat more darkly for ATPase, pH 9.4 than the type I fibers in the peripheral region.

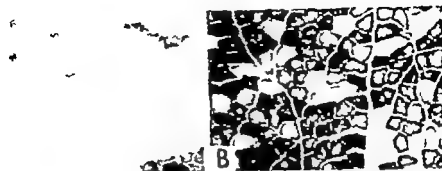


Fig. 1. Serial cross sections through central region of longissimus at level of 5th lumbar vertebra. A, $\text{NADH}_2\text{-TR}$, pH 9.4; B, $\text{NADH}_2\text{-TR}$.

II A and II B 56 μm) whereas all the fibers in the central region are about equal in size (I 54 μm , type II 56 μm).

Specimens obtained from superficial as well as central regions also at other levels along the muscle were analyzed with respect to fiber composition. Superficial portions at the levels from 7th to the 2nd lumbar vertebrae and specimens collected from the central region along the well developed intermuscular septum (cf. Carlson 1978 Fig. 3 D-G) exhibit about the same fiber composition as that of the two regions at the level of the 5th lumbar vertebra. Vertical bundles at the level of the first lumbar vertebra are composed of up to 20% of type I fibers and about 14% of type II A fibers. Hence, type II B fibers are less numerous in this part of the muscle. Also specimens obtained from other deep regions than the central part of the muscle exhibited this composition of fiber types. In summary the peripheral portion of the longissimus muscle is mainly composed of type II fibers with a low oxidative ability while the central part, connected to the most medial division of the prominent intermuscular septum, has a high proportion of type I fibers.

The dissections. In specimens obtained from superficial parts of the muscle at the level of the 5th lumbar vertebra type I fibers represent about 5%, type II A 13% and type II B 82%.

5th lumbar back muscles

No. med.		Intermediate med.			Multifidus			Interspinales		
II A	II B	I	II A	II B	I	II A	II B	I	II A	II B
22	13	12	23	65						
		17	76	57	26	24	30			
1	28	26	70	54	28	23	49	40	13	47
15	28	23	25	52	22	16	62	36	14	30
46	48	38	43	83	47	47	58	43	45	51
8.4	9.1	7.5	9.9	12.8	5.4	6.4	8.0	6.7	6.7	9.5

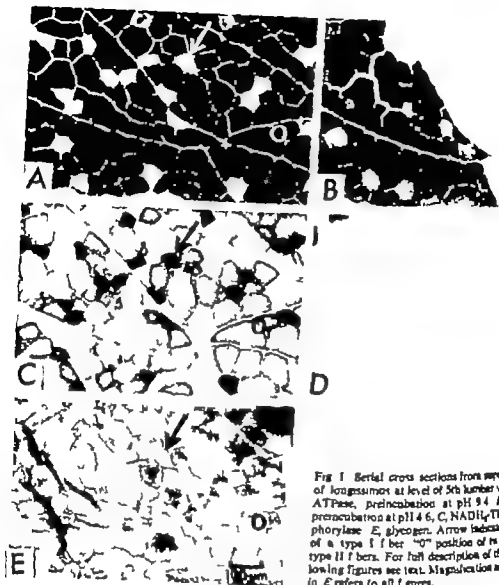


Fig. 1. Serial cross sections from superficial of longissimus at level of 5th lumbar vertebra. ATPase, preincubation at pH 9.4, A, ATP preincubation at pH 4.6, C, NADH-TR, and phosphorylase, E, glycogen. Arrow indicates position of a type I fiber. "0" position of a deltoid type II fiber. For full description of this and following figures see text. Magnification as indicated in E refers to all figures.

TABLE III. Frequency in percentages, mean diameter (\bar{x}) and standard deviation (s) in μ m of type

Cat no.	Longissimus L_4 superficial			Longissimus L_4 central		Iliocostalis L_4 superficial			Sacrocaudalis dors. lat.	
	I	II A	II B	I	II	I	II A	II B	I	II A
1	5	16	79			1	15	84		
2	8	12	80			4	7	89	8	13
3	6	13	81			4	13	83	7	14
4	12	17	71			8	14	78	19	17
5	11	12	77	66	34	13	80	18	18	14
6	11	13	76	72	28	9	14	77		
fiber diameter ^a										
\bar{x}	38	45	57	54	56	35	42	52	45	50
s	6.1	9.5	14.4	7.8	8.2	4.9	8.3	10.7	7.1	7.5

^a Methods, data obtained from cat no. 5.

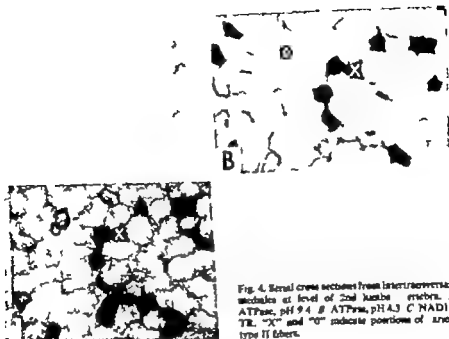


Fig. 4. Serial cross sections from intertransversarii medullae at level of 2nd lumbar vertebra. A, ATPase, pH 9.4; B, ATPase, pH 4.3; C, NADH-TR; D, SDH; E, fat. "X" and "O" indicate portions of atrophic type II fibers.

the type II fiber population are obvious. The type II (fibers in the upper part of the muscle (Fig. 4) exhibit a broader spectrum of staining intensities which is exemplified by the three type II fibers indicated by an "X" in the figure. The lowermost of these three fibers shows weak reaction (type II B) while the upper two display different degrees of intermediate reactions (type II A). Fibers of the latter type constitute about 23% of the population at this level and thus type II B fibers amount to 57%. The diameters of the various type II fibers also differ markedly in this region (see Table III).

Biopsies also from other levels along the lumbar part of the muscle (L_2 , L_3 , L_4) were analyzed with respect to fiber composition. A gradual increase of the proportion of the type I fibers and corresponding decrease of the type II B fibers towards the lower levels of the muscle are apparent.

The multifidus. Serial cross sections at the level of the 5th lumbar vertebra are shown in Fig. 5. Type I fibers, lightly stained in A (ATPase, pH 9.4) and darkly in B (ATPase, pH 4.6), constitute about 25% of the fiber population. Type II fibers, darkly stained in A, exhibit several gradations in the stainability for NADH-TR (C), SDH (D) and fat (E). The type II A fibers, reacting low in B and intermediate or high in C, D and E, form about 21% of the fibers. The type II B fibers, reacting intermediate in B and with a low stainability for the oxidative enzymes and fat, constitute about 54% of the fiber population. In the figure two type II fibers representative of the two subtypes are indicated by an "O". Specimens collected from other levels along the lumbar region have a similar fiber composition. From the figure it is also evident that the type I and type II A fibers are small whereas type II B fibers are considerably larger (see Table III).



Fig. 3 Serial cross sections from intertransversarii mediales at level of 5th lumbar vertebra. A. ATPase, pH 9.4. B. NADH-TR. "O" indicates position of two different type II fibers.

Analysis of the fiber composition at other levels along the muscle (L_4-L_5) did not reveal great deviations from these values. However muscle bundles in the uppermost lumbar region inserting onto the ribs as well as deeper portions of the muscle are made up of the 20% of type I fibers, 15% of type II A and 65% of type II B fibers. Hence, the superficial and deeper regions of the iliocostalis have a fiber composition similar to that of the peripheral part of the longissimus. The diameters of the various fiber types in the superficial portions correspond to those of the fibers in the superficial longissimus muscle (cf. Table II). In deep regions the type I fibers are somewhat larger than in superficial parts whereas the sizes of the various type II fibers are about the same in the two regions of the iliocostalis muscle.

The sacrocaudalis dorsalis lateralis In this muscle the type I fibers constitute about 15%, type II A 17% and type II B 70%. However there are larger interindividual variations in fiber composition in this muscle than in the other lumbar back muscles (see Table III). The staining reactions as well as the sizes of the various fiber types correspond to those of the fiber population for example in the superficial parts of the longissimus muscle.

The intertransversarii mediales In Fig. 3 are shown sections of the muscle at the level of the 5th lumbar vertebra. Type I fibers lightly stained for myofibrillar ATPase, pH 9.4 (A) constitute about 60% of the fibers. Various type II fibers are seen in the sections stained for NADH-TR (B). It is obvious that the fibers exhibit either an intermediate (type II A) or a weak (type II B) reaction for the oxidative enzyme. Representative of the two subtypes are the fibers indicated by an "O" in the figure. In this part of the muscle, types II A and II B constitute about 17% and 23% respectively of the total fiber count. The individual fibers are very similar in size and the diameters are estimated at 47 μ m in type I, 46 μ m in type II A and 48 μ m in type II B.

Fig. 4 illustrates another part of the same muscle, at the level of the 2nd lumbar vertebra. This part of the muscle differs in several respects from that in Fig. 3. Type I fibers appear light in the section stained for myofibrillar ATPase after preincubation at pH 9.4 (A) and dark when stained for ATPase, pH 4.3 (B) constitute only about 20% of the fiber population. The diameter of the type I fibers is also lesser than in the lower part of the muscle (see Table III). When comparing the sections stained for NADH-TR (Fig. 4 C) differences

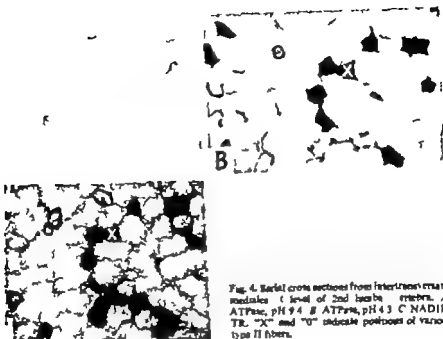


Fig. 4. Serial cross sections from intertransverse muscle (level of 2nd lumbar vertebra). A, ATPase, pH 9.4; B, ATPase, pH 4.3; C, NADH-TR. "X" and "U" indicate positions of various type II fibers.

The type II fiber population are obvious. The type II fibers in the upper part of the muscle (Fig. 4) exhibit a broader spectrum of staining intensities which is exemplified by the three type II fibers indicated by an "U" in the figure. The lowermost of these three fibers shows a weak reaction (type II B) while the upper two display different degrees of intermediate reactions (type II A). Fibers of the latter type constitute about 23% of the population at this level and thus type II B fibers amount to 37%. The diameters of the various type II fibers also differ markedly in this region (see Table II).

Specimens also from other levels along the lumbar part of the muscle (L_2 , L_3 , L_4) were analyzed with respect to fiber composition. A gradual increase of the proportion of the type I fibers and a corresponding decrease of the type II B fibers towards the lower levels of the muscle were apparent.

The multifidus. Serial cross sections at the level of the 5th lumbar vertebra are shown in Fig. 5. Type I fibers, lightly stained in A (ATPase, pH 9.4) and darkly in B (ATPase, pH 4.6), constitute about 25% of the fiber population. Type II fibers, darkly stained in A, exhibit several gradations in the stainability for NADH-TR (C), SDH (D) and fat (E). The type II A fibers, reacting low in B and intermediate or high in C, D and E, form about 21% of the fibers. The type II B fibers, reacting intermediate in B and with a low stainability for the oxidative enzymes and fat, constitute about 54% of the fiber population. In the figure two type II fibers representative of the two subtypes are indicated by an "U". Specimens collected from other levels along the lumbar region have a similar fiber composition. From the figure it is also evident that the type I and type II A fibers are small whereas type II B fibers are considerably larger (see Table III).

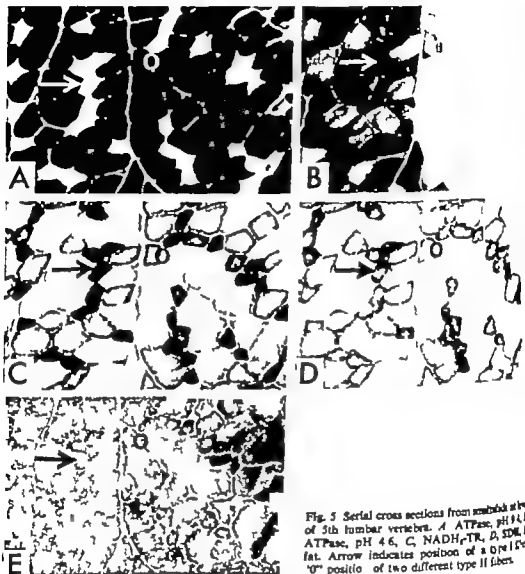


Fig. 5 Serial cross sections from multifidus of 5th lumbar vertebra. A ATPase, pH 9.4, B ATPase, pH 4.6, C, NADH-TR, D, SDH, E fat. Arrow indicates position of a fiber. '0' position of two different type II fibers.

The interspinales These bundles lie just medial to the multifidus and have a different fiber composition which is evident when comparing Fig. 5 (multifidus) and Fig. 6 (interspinales L₅-S₁). Type I fibers, lightly stained in Fig. 6 A (ATPase, pH 9.4) constitute about 38% of the fiber population. Type II fibers, appearing dark in A show low to high stainability in B (NADH-TR). Those reacting intermediate or high (type II A) make up about 14% and the pale fibers (type II B) 48% of the total population. Specimens from other interspinal spaces were also analyzed and displayed only minor variations in fiber composition as specified above.

When comparing the fiber composition of different lumbar back muscles it is obvious that there are large differences in the proportions of type I and type II B fibers whereas the differences in the proportion of type II A fibers are less pronounced. Muscle spindles are found in all the muscles investigated. An analysis of the



Fig. 1. Serial cross sections through intervertebral discs between spinous processes of 4th and 5th lumbar vertebrae. A, ATPase, pH 9.4; B, NADH₂ TR.

les in the various back muscles has not been made but a specific pattern of spindle pattern in the longissimus muscle has been observed. In the peripheral part of the muscle, made up of a high proportion of type II fibers, the spindles are very sparse whereas they are numerous in the central part which is mainly composed of type I fibers.

Discussion

One of the aims of this investigation was to characterize the lumbar back muscle fibers histochemically and to relate the fiber composition of the various muscles to their contraction times as described in a preceding paper (Carlson 1978).

The starting pattern and the classification of the muscle fibers have been considered previously. It has previously been shown that the biochemically estimated activity of myofibrillar ATPase in smooth and striated muscles is higher the shorter the contraction time of the muscle (Birley 1967). With a histochemical mapping procedure (Edström and Kugelberg 1971) it has also been shown that fast-twitch units in the rat soleus muscle (Kugelberg 1971) as well as in the cat gastrocnemius muscle (Burke et al. 1971, 1973) are composed of units with a high activity of myofibrillar ATPase (type II) and that slow-twitch units are made up of fibers with low stainability for the enzyme (type I).

The data obtained in the present study show that a very high proportion of the fibers in longissimus and in the iliocostalis muscles has a high activity of myofibrillar ATPase. The proportion of such fibers is lower in the medial muscles multifidus and interspinales. In a study of the contraction properties the lateral muscles longissimus and iliocostalis were analyzed separately, nor were the two medial muscles multifidus and interspinales. The question then arises whether the contraction times and the fatigue properties observed are representative of the individual muscles in view of their fiber composition. The multifidus makes up the main portion of the medial muscles while the interspinales constitute a very small part and hence the mean contraction time, 34 ms, is representative of the multifidus but not necessarily of the interspinales. The latter muscles may in fact, be expected to contract more slowly since their proportion of type II fibers is lower than in the multifidus.

Considering the small differences in the fiber composition of the two lateral muscles longissimus and iliocostalis it is apparent that the average time to peak tension, 29 ms, is

representative of both muscles. However the previous study also revealed a slow component with a contraction time of about 100 ms located in the middle and lower parts of the longissimus muscle. This fits in with the findings in the present investigation. The central part of the longissimus, located in close connection to the intermuscular septum, is made up of a high proportion of type I fibers. These are considerably larger than the type I fibers at the periphery of the muscle and stain somewhat darker for myofibrillar ATPase, pH 9.4. In addition, in stainings for oxidative enzymes the stained products are not as tight as in other type I fibers. Histochemically these fibers are very similar to cat soleus I (cf. Nyström 1968, Burke *et al.* 1974). It is possible that the type I fibers in the central part of the longissimus represent a unique population relative to other type I fibers in the muscles. To provide further information on this point a comparative analysis of all motor units is however necessary.

In the central region of the longissimus, muscle spindles are more frequent than in other parts of the muscle. An association between spindles and muscle fibers presumed to be of slow twitch type is present in other muscles as well (see e.g. Richmond and Abrahams 1975). The findings indicate that the proprioceptive reflex control differs in various parts of the muscle.

In the longissimus and the iliocostalis, the predominant fiber type has a low staining for oxidative enzymes and fat but stains more readily for phosphorylase and glycogen (type II B). Fibers with this histochemical profile are apt to be depleted of their glycogen on prolonged stimulation and the motor units are found to be less resistant to fatigue. Motor units composed of fibers with a higher activity of oxidative enzymes (Kugelberg and Nyström 1968, Edström and Kugelberg 1968). On the other hand, in the multifidus and the interspinales muscles about 50% of the fibers have an intermediate or high staining for oxidative enzymes. Thus, one would expect the medial muscles to be considerably more resistant to fatigue than the longissimus and the iliocostalis. However this is not the case. The difference in performance between the two groups of muscles is small: the lateral muscles retain about 13% of their initial tension after a period of 10 min of low-frequency stimulation while the corresponding value for the medial muscles is 21% (Carlson 1977). In these experiments, signs of a failure in the impulse propagation were observed for both the lateral and the medial muscles. This indicates that the observed decline in contraction tension may not exclusively be attributed to exhaustion of energy stores in the muscle fibers.

The results obtained in the previous investigation of the morphology and contraction properties of the lumbar back muscles demonstrated a functional differentiation between the various muscles. By the present analysis of their enzyme activity and substrate content this differentiation has been further clarified.

This work was supported by a grant from the funds of Karolinska Institute.

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representative of both muscles. However the previous study also revealed a slow core with a contraction time of about 100 ms located in the middle and lower parts of the biceps muscle. This fits in with the findings in the present investigation. The central part of the longissimus, located in close connection to the intermuscular septum, is made up of a high proportion of type I fibers. These are considerably larger than the type I fibers at the periphery of the muscle and stain somewhat darker for myofibrillar ATPase, pH 9.1. In addition, in stainings for oxidative enzymes the stained products are not as tightly packed as in other type I fibers. Histochemically these fibers are very similar to cat soleus fibers (cf Nyström 1968 Burke *et al* 1974). It is possible that the type I fibers in the central part of the longissimus represent a unique population relative to other type I fibers in the lumbar muscles. To provide further information on this point a comparative analysis of motor units is however necessary.

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Anthropometric measurements. The following anthropometric measurements were performed: body and height, femur-condyle and radio-ulnar widths and skinfolds of subscapula, triceps brachii, biceps, and suprapubic area (Dorset and Makinon 1967). For correlative analysis, fat-free body mass was estimated according to von Döbeln (1959).

Chair power was determined by running maximally for a few seconds in a staircase as described by *van et al.* (1966). For further details see *Korn et al.* 1977.

Isometric forces were measured in maximal voluntary isometric extensions of the right knee (*quadriceps*) and of both legs (*total leg force*), respectively. *Force-time* curve was registered during the total leg measurements and the time to reach 70% of the maximum force was taken as *force-time value* (for *Therrien et al.* 1976).

Isometric electromyographic activity (IEMG) was picked up from both *an. rectus femoris* and *an. vastus* during maximum right knee extension (Vittalo and Korn 1975).

Isometric measurements were obtained from the *vastus lateralis* muscle in the constant current of the following stimulus durations: 0.1 ms, 1 ms and 30 ms. The 30 ms duration is considered to be the maximal and is therefore used as basis for calculation of the chronaxie value.

Peak power *lateralis* maximum oxygen uptake ($V_{O_2 \text{ max}}$) as determined during treadmill running according to *Belton and Astrand* (1967). The Douglas bag technique as used for collection of expired air. O_2 and CO_2 contents were measured on Schöndler gas analyzer.

Heart rate (HR) as recorded during the $V_{O_2 \text{ max}}$ test and the HR-value at the termination of the run taken as the peak heart rate.

Blood lactate concentrations as determined in arterialized finger tip blood samples taken 3 to 5 min after the maximal treadmill run. Reagents and instructions of Boehringer Mannheim GmbH were for the determination of the whole blood lactate concentrations.

Muscle biopsy specimens. Two muscle biopsy samples were taken from the *vastus lateralis* muscle (method by *Bergstrom* (1962)). One biopsy sample as used for classification of muscle fibres into fast (FT) and slow (ST) types (*Gollnick et al.* 1972) by staining for ATPase as instructed by *Isaacs and Huxley* (1955).

Enzyme assays. The second biopsy sample as used for determination of enzyme activities. The enzymes assayed were Mg^{2+} stimulated ATPase, creatine phosphokinase (CPK), and myokinase (MK) according to *Therrien et al.* (1976), lactate dehydrogenase (LDH) according to *Spöck* (1976), phosphorylase (P), hexokinase (HK) and Ca^{2+} stimulated ATPase (*Lowry and Passonneau* 1972). Distribution of the muscle specific LDH isoenzyme, LDH-4, was determined in the muscle biopsy specimens as described *Karlsson et al.* (1974).

Statistical analysis. Ordinary statistical procedures have been employed to calculate the means, standard deviation (SD) and correlation coefficients (r).

Results

Table 1 summarizes the comparison in the different variables between male and female subjects.

Anthropometric variables. The major differences in body size between females and males are present in weight, height (for both variables larger values in the males), skinfold data (higher in the females) and fat percent (higher in the females) (Table 1).

Performance variables. $V_{O_2 \text{ max}}$ was higher in the males both in $l \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ and $\text{ml} \cdot \text{min}^{-1}$ (Table 1). Peak blood lactate tended also to be higher in the males.

Of the different strength and neuromotoric (including IEMG) variables studied the results of the females were from 51.7–84.6% of that of the males. Especially pronounced was the difference in times to reach the 70% isometric force level, which was almost twice (198.9%) longer in females. In chronometric measurements no or only minor differences were present between the sexes (Table 1).

Muscle characteristics. The males tended to have a higher %ST fibre distribution

Skeletal muscle fibre types, enzyme activities and physical performance in young males and females

By

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Abstract

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Differences in skeletal muscle characteristics, metabolic profiles and functional performance between males and females were investigated using young (15-24 yrs) male and female twins as subjects. The comparison included such variables as anthropometry, muscle strength, mechanical power, maximum oxygen uptake, electrical activation of muscle, muscle fibre composition (in vastus lateralis), and activities of several skeletal muscle enzymes. The results disclosed the following primary differences between males and females: 1. In the various functional tests the performance of females was from 61.1 to 84.6% of that of males. 2. The distribution of slow twitch fibres in m. vastus lateralis of the females ($49.1 \pm 7.7\%$) was lower ($p < .05$) than that of the males (55.9 ± 11.9). 3. Activities of enzymes Ca⁺⁺-stimulated ATPase, CPK, phosphorylase, LDH₁ and LDH₅ were higher ($p < .05-.01$) in the males, whereas the distribution pattern of LDH₁ isoenzymes was higher ($p < .05$) in the females. A pronounced difference between the two groups was a shorter relaxation time of isometric force in females. It is concluded that the males as compared to the females demonstrate higher aerobic and strength performance capacity, more efficient neuromotoric control of contraction, more slow twitch muscle fibres and more pronounced contractile and glycolytic potential of the skeletal muscles.

With the exception of a recent preliminary report (Hedberg and Jansson 1976) the knowledge is scarce concerning possible differences in muscle characteristics and metabolic profiles between males and females. The present study is based on data collected in an examination of monozygous and dizygous twins of both sexes (Komi *et al.* 1976 and Komi *et al.* 1977) in respect to histological, histochemical and physical performance variables.

Methodology

Subjects for the study were obtained through the Population Register of Finland. They were all serologically tested according to routine methods (for ref. Komi *et al.* 1976). The final sample was composed of 10 male (9 MZ and 11 DZ) and 11 female (6 MZ and 5 DZ) twin pairs, i.e. approximately a similar distribution of MZ and DZ pairs among males and females. With the exception of one male pair (11 years) ages ranged from 15 to 24 years with the means of 17.8 and 19.4, respectively for males and females.

Table 2. Summary of the correlation matrix for performance variables showing the significant ($p < .01$) correlation coefficients for females (left) and males (right).

	$\dot{V}O_{2\max}$ l min ⁻¹	$\dot{V}O_{2\max}$ ml kg ⁻¹ min ⁻¹	LA	RV	MP	TLF	QF	FT	% ST
Females									
$\dot{V}O_{2\max}$ (l min ⁻¹)	■								
$\dot{V}O_{2\max}$ (ml kg ⁻¹ min ⁻¹)	■	■							
Blood lactate (LA)			■						
Running velocity (RV)				■					
Muscular power (MP)					■				
Total leg force (TLF)						■			
Quadriceps force (QF)							■		
Force-time (FT)								■	
ST fibres (% ST)									■
Males (MZ + DZ)									
$\dot{V}O_{2\max}$ (l min ⁻¹)					.33	.46	.69		
$\dot{V}O_{2\max}$ (ml kg ⁻¹ min ⁻¹)									
Blood lactate (LA)									
Running velocity (RV)									
Muscular power (MP)									
Total leg force (TLF)									
Quadriceps force (QF)									
Force-time (FT)									
ST fibres (% ST)									

in the females ($p < .05$) but also a large range of variation as expressed by ± 1 S.D. which is 11.9 in the males as compared to 7.7 in the females.

Approximately similar muscle enzyme activities were obtained for males as compared to females in respect to MK, HK, LDH in the forward reaction ($LDH_{pp \rightarrow ss}$), MG and stimulated ATPases, CPK, Phos, and LDH in the backward reaction ($LDH_{ss \rightarrow pp}$) are higher (15–67%) in the males, whereas the distribution pattern of LDH-i demonstrated higher percentage in the females (55%).

Separate analysis

For the purpose of comparative analysis correlation matrices were formed to obtain interrelationships between all the variables studied. In the following only the most relevant information can be presented. For details, the reader is referred to Komi and Karlsson 1978.

Maximal oxygen uptake ($\dot{V}O_{2\max}$) expressed in l min⁻¹ was in the male material best correlated to the following variables: muscular power ($r = .83$), quadriceps force ($r = .69$) and total leg force ($r = .46$) (Table 2). Similar correlations were obtained in the female material with the exception of quadriceps force. When $\dot{V}O_{2\max}$ was expressed per kg b.wt. in the males the best correlations were found to variables of peak blood lactate ($r = .56$) and running velocity ($r = .57$). In females skin variable was related to force-time ($r = -.56$) and in contrast to the males even to the alternate way to express aerobic power (l min⁻¹ $r = .66$).

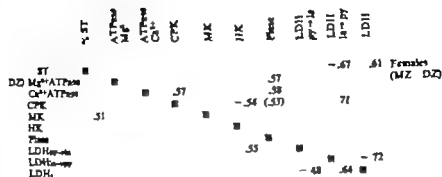
Muscular power was in the two materials best related to $\dot{V}O_{2\max}$ expressed as l min⁻¹ ($r = .83$ in both sexes, respectively) and running velocity ($r = .54$ in both sexes). No significant

TABLE 1 Means \pm S.D. of the different variables for the monozygous (MZ) and dizygous (DZ) twins both sexes.

	Females MZ+DZ	Males MZ+DZ	Females/ males* (per cent)	t	P
1 Anthropometric variables					
Weight (kg)	50.8 \pm 7.7	62.6 \pm 12.7	81.2	3.99	<.01
Height (cm)	161.7 \pm 6.7	175.9 \pm 6.9	91.9	8.19	<.01
Femur-condyle II	8.2 \pm 0.3	9.5 \pm 0.4	86.3	-12.17	<.01
widths (cm), rt	8.2 \pm 0.3	9.4 \pm 0.5	87.2	-11.60	<.01
Radio-ulnar II	4.9 \pm 0.2	5.7 \pm 0.3	86.0	-9.88	<.01
widths (cm), rt	4.9 \pm 0.2	5.7 \pm 0.4	86.0	8.06	<.01
Scapula skinfold (mm)	9.9 \pm 3.5	8.6 \pm 4.4	115.1	1.20	n.s.
Triceps skinfold (mm)	13.2 \pm 3.5	7.0 \pm 3.3	188.6	6.90	<.01
Biceps skinfold (mm)	5.1 \pm 1.1	3.7 \pm 2.0	137.8	2.69	<.05
Crista ilica skinfold (mm)	6.7 \pm 3.4	7.6 \pm 5.0	88.2	0.84	n.s.
Fat (%)	22.5 \pm 3.2	11.2 \pm 4.8	200.9	9.85	<.01
2. Performance variables					
VO ₂ max (l \times min ⁻¹)	2.2 \pm 0.5	3.6 \pm 0.7	61.1	-8.40	<.01
VO ₂ max (ml \times /kg \times min ⁻¹)	43.2 \pm 4.7	56.6 \pm 5.7	76.3	-9.42	<.01
Peak heart rate	190.0 \pm 8.7	195.0 \pm 11.8	97.4	-1.74	n.s.
Peak blood lactate	6.4 \pm 2.2	8.8 \pm 2.6	72.7	3.68	<.05
Running velocity (m \times s ⁻¹)	1.1 \pm 0.1	1.3 \pm 0.1	84.6	8.73	<.01
Muscular power (kpm \times s ⁻¹)	56.6 \pm 10.4	82.9 \pm 17.7	68.3	-6.53	<.01
Total leg force (kp)	171.9 \pm 30.0	214.2 \pm 54.5	80.3	-3.53	<.05
Quadriceps force (kp)	40.3 \pm 9.8	56.8 \pm 15.5	71.0	-4.67	<.01
Force-time (ms)	748.1 \pm 344.2	376.3 \pm 255.7	198.9	5.91	<.01
3 Muscle fibre composition					
ST fibres (%)	49.1 \pm 7.7	55.9 \pm 11.9	87.8	-2.49	<.05
4 Muscle enzyme activities (moles \times g ⁻¹ min ⁻¹ \times activity constant)					
Mg ²⁺ ATPase 10 ⁻⁶	8.7 \pm 2.6	10.0 \pm 3.5	87.0	-1.20	n.s.
Ca ²⁺ ATPase 10 ⁻⁶	0.06 \pm 0.01	0.1 \pm 0.1	60.0	-2.11	<.05
CPK 10 ⁻⁶	0.02 \pm 0.01	0.03 \pm 0.01	66.7	-3.17	<.01
MK 10 ⁻⁶	80.6 \pm 23.7	74.2 \pm 23.6	108.6	0.34	n.s.
HK 10 ⁻⁶	0.10 \pm 0.02	0.10 \pm 0.04	100.0	-0.48	n.s.
Phase 10 ⁻⁶	0.70 \pm 0.4	1.05 \pm 0.6	66.7	-2.21	<.05
LDH _{pp-ss} 10 ⁻⁴	1.50 \pm 0.6	1.45 \pm 0.6	103.4	0.36	n.s.
LDH _{ss-ss} 10 ⁻⁴	0.50 \pm 0.2	0.70 \pm 0.2	71.4	-2.18	<.05
LDH I isozyme (%)	25.3 \pm 18.3	16.3 \pm 12.2	155.2	2.23	<.05
5 Electromyographic variables					
EMG, rectus femoris (mV \times s ⁻¹)	498.1 \pm 225.9	608.1 \pm 199.2	81.9	-1.63	n.s.
EMG, vastus lateralis (mV \times s ⁻¹)	281.4 \pm 165.5	544.6 \pm 408.3	51.7	-3.77	<.01
6. Chronaximetric variables					
30 ms	4.0 \pm 1.0	3.9 \pm 1.4	102.6	-0.03	n.s.
1 ms	5.3 \pm 1.0	5.5 \pm 1.9	96.4	-0.14	n.s.
0.1 ms	16.5 \pm 5.1	19.9 \pm 13.8	82.9	0.78	n.s.

* Mean per cent values of the females as compared to the males.

III. Summary of the correlation matrix for muscle biopsy variables showing the significant ($p < 0.1$) correlation coefficients for males (left) and females (right).



T was related significantly only to MK activity ($r = -0.51$). This relatively high value and the same relationship to be significant also in the total material (Fig. 2). In females T was related significantly to LDH_{iso-1} and % LDH 1 (Table III, Fig. 3 and 4), respectively with $r = 0.67$ and $r = 0.61$.

Discussion

The present study confirms the results of earlier studies that the males had a higher physical performance capacity than the females. In respect to aerobic performance this was early demonstrated and explained among others by Astrand (1952). The difference in strength performance has not been as extensively investigated although the fact that the muscle mass is larger in males has been pointed out as one contributory factor (Hermansen and von Döbeln 1971). In this connection it is important to emphasize the role of total mass determining the force production, because the force per unit of cross-sectional area of muscle is similar regardless of sex or to some extent of training (Ikai and Fukunaga 1968).

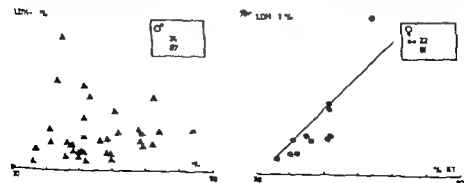


Fig. 3. Relationship between the distribution of isoenzyme LDH-1 and distribution of the slow twitch fibres of the vastus lateralis muscle in male (left) and female (right) subjects.

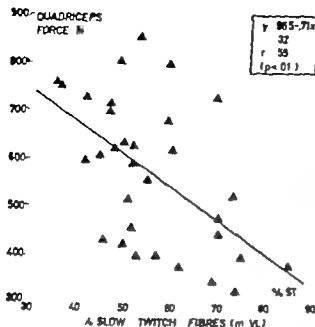


Fig. 1 Relationship between isometric force of the quadriceps muscle group (last session) and per cent distribution of slow twitch fibres (ST_m) of the vastus lateralis from all male subjects.

relationship was, however present between muscular power and percent distribution of ST fibres neither in males nor females. In addition, muscular power demonstrated in both sexes a high correlation to percent fat ($r = .66$ and $.73$ in the females and males, respectively).

Quadriceps force demonstrated in correlative analysis further differences between the sexes (Table II). Thus, in males total leg force ($r = .82$), muscular power ($r = .82$), \dot{V}_{O_2} ($l \times min^{-1}$, $r = .69$), percent ST fibres ($r = -.55$ Fig. 1) showed the highest correlations to quadriceps force.

Muscle fibre type distribution and enzyme activities Two major sex differences were observed when % ST fibres were correlated with the various muscle enzyme activities. In males

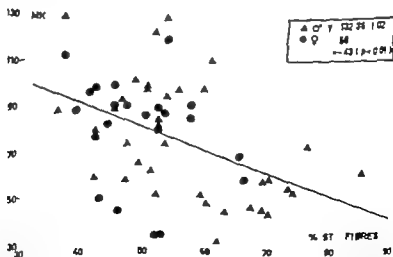


Fig. 2 Relationship between the activity of myokinase enzyme (MK) and per cent slow twitch fibres of the vastus lateralis muscle in all subjects including males and females. MK activity is expressed as $mg^{-1} \times min^{-1} \times 10^{-3}$.

for lactate oxidation the per cent distribution of LDH-H isozyme was higher in the males. Normally quantitative and qualitative shifts in LDH isozyme patterns are observed in relation to endurance training (Sjodin 1976). However it seems unreasonable to use training adaptation as an explanation for the observed sex differences. On the other hand the mechanisms operating during or initiated by training resulting in similar LDH isozyme shifts are also unknown.

Qualitative differences between male and female skeletal muscle in terms of enzyme activities explain partly the observed differences in the force-time curves. It is likely that factors might also be involved in causing the difference in the force-time characteristics in males and females. It has already been demonstrated that the elastic behavior of the tendons are different in males and females (Komi and Bosco 1977). It seems that the females more efficiently utilize the elastic energy which is stored in the activated muscle during prestretching. However they were relatively poor in jumping movement, which demands from a static starting position that allows practically no storage of elastic energy. Again, however it is difficult to state whether these differences between the sexes are explainable by hormonal factors or training status. Irrespective the causal background, the relationship between force-time and muscular power for both sexes ($r = -0.59$ and -0.31) might indicate how the different variables are integrated in performance. The lower isometric efficiency (represented by force-time) in combination with a qualitatively and quantitatively different skeletal muscle mass will result in a lower muscular power in the females.

Summary the males demonstrated as compared to the females the following differences:

- higher aerobic performance capacity
- higher strength performance capacity
- more efficient neuromotoric output during muscle contraction
- more ST muscle fibres as well as larger individual variations in their skeletal muscles
- more pronounced contractile and glycolytic profiles in their skeletal muscles

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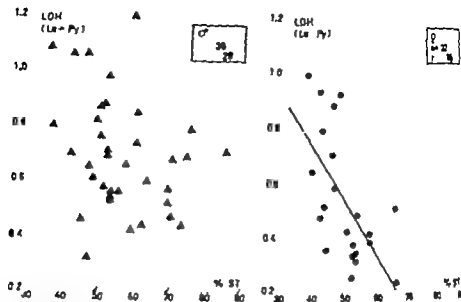


Fig. 4. Relationship between the activity of enzyme LDH_{L+Py} and distribution of the slow muscle fibres in the vastus lateralis muscle in male (left) and female (right) subjects.

If the total leg force in the present study is related to the body weight (then the mean values are almost identical for the two groups 3.38 (females) and 3.40 (males).

The most important contribution from the present study is the differences between sexes in terms of qualitative aspects on skeletal muscle. That the muscle fibre distribution is different has already been pointed out independently by a parallel investigation on men and females (Hedberg and Jansson 1976). The means were 53 ± 12.2 (S.D.) and 49 (S.D.) percent ST fibres, respectively, i.e. that not only the means are different between sexes but the males have also slightly larger individual variation in muscle fibre composition. In addition to a higher percent ST fibres the males had also higher values for ATPase of CPK (Table I). These enzymes are normally more characteristic for the metabolic profile of the FT fibre (Gollnick *et al.* 1974 and Thorstensson 1976).

The glycolytic profile seems also to be different and higher in the males. Previous studies with testosterone on castrated rats have demonstrated an increase in phosphorylase activity which is in line with the present data (Karlsson *pers. com.*). Further support for the concept of a more pronounced glycolytic profile in male skeletal muscle is given by the study of Hedberg and Jansson (1976), who demonstrated phosphofructokinase (PFK) activities corresponding to 15.69 ± 4.72 (S.D.) and 13.18 ± 3.06 (S.D.) in males and females, respectively. The more pronounced glycolytic profile in males is also emphasized by the fact that the males have a larger percentage of ST fibres. Providing the samples have been obtained from a homogeneous material the FT fibres normally possess higher contractile and glycolytic characteristics (Thorstensson 1976 and Sjødin 1976). The reason why the females differ in respect to qualitative aspects on skeletal muscle is unclear. Although hormonal influences are indisputable as contributory factors, adaptation processes due to different physical activity patterns cannot be excluded.

In respect to the glycolytic profile it is of interest to note that in spite of the lower LDH

for lactate oxidation the per cent distribution of LDH-I isozyme was higher in the

Normally quantitative and qualitative shifts in LDH isozyme patterns are observed in relation to endurance training (Sjödén 1976). However it seems unreasonable to tracing adaptation as an explanation for the observed sex differences. On the other hand mechanisms operating during or initiated by training resulting in similar LDH shifts are also unknown.

Qualitative differences between male and female skeletal muscle in terms of enzyme composition explain partly the observed differences in the force-time curves. It is likely that factors other than enzyme composition are also involved in causing the difference in the force-time characteristics in males and females. It has e.g. been demonstrated that the elastic behavior of the tendons are different in males and females (Komi and Bosco 1977). It seems that the females use more efficiently the elastic energy which is stored in the activated muscle during prestretching. However they were relatively poor in e.g. jumping movement, which is based on a static starting position that allows practically no storage of elastic energy. Again, however it is difficult to state whether these differences between the sexes are explainable by hormonal factors or training status. Irrespective the causal background, the relationship between e.g. force-time and muscular power for both sexes ($r = -0.59$) might indicate how the different variables are integrated in performance. The lower motoric efficiency (represented by force-time) in combination with a qualitatively and quantitatively different skeletal muscle mass will result in a lower muscular power in the females.

In summary the males demonstrated as compared to the females the following differences:

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- higher strength performance capacity
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The release of serotonin from rat duodenal enterochromaffin cells by adrenoceptor agonists studied *in vitro*

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Abstract

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The release of serotonin from rat duodenal enterochromaffin cells by adrenoceptor agonists studied in vitro. Acta physiol. scand. 1978, 103, 219-224.

The serotonin (5-HT) content of enterochromaffin cells (EC) as studied by cytofluorimetric method is given from rat duodenal mucosa after *in vitro* incubation with different adrenoceptor agonists and agonists and acetylcholine (ACh). Norepinephrine (NA), Adrenaline (A) and Isoprenaline (IP) caused release of 5-HT in EC dose to 40-60% and for NA and A this effect was concentration-dependent. The effect was antagonized by α_1 -propranolol but not by α_2 -propranolol, metoprolol, phentolamine or phenoxybenzamine indicating that the 5-HT release from EC is probably mediated via true β -adrenoceptor receptors. Addition of the β_2 type ACh also decreased the 5-HT content of EC but was much less potent than the adrenergic substances. Dopamine (DA) had no effect.

Key words: Rat enterochromaffin cells—duodenal mucosa—*in vitro*—serotonin release—cytofluorimetry— β -adrenoceptor.

The enterochromaffin cells (EC) in mammals, such as mouse, rat, guinea pig, cat and man, has been shown to contain serotonin (5-HT) stored in large granules in the cytoplasm (Jenden and Wong 1957, Penttilä 1966). Populations of these cells have recently been demonstrated immunohistochemically to also store polypeptides, e.g. motilin and substance P (Pearse *et al.* 1974, Hertz *et al.* 1976, Polak *et al.* 1976). Thus, the EC of the gut fulfil the criteria of the so called APUD-group of endocrine cells, which can take up amine precursors, decarboxylate them and store the amine together with the hormonal peptide (*cf.* Pearse 1969).

The functional control of the EC in the gut is unknown but previous studies have suggested different possible mechanisms. 1) *Intraluminal stimuli*, such as instillation of hypertonic glucose or acidification of the duodenum cause degranulation of EC and concomitant release of 5-HT to the blood (Drapanas *et al.* 1962, Fujita and Kobayashi 1971, Kellum and 1976). 2) *Substances in the circulation* e.g. norepinephrine (NA) infusion to dog isolated

intestine, cause release of 5-HT into the perfusate (Burks and Long 1966). 3) Vagus may control release from the EC. For instance, stimulation of the cervical vagal nerve in cats causes a decrease of 5-HT in the EC of the small intestine (Ahlman *et al.* 1976a). This was demonstrated by a direct cytofluorimetric technique where the 5-HT content in individual EC was estimated in samples taken before and after cervical nerve stimulation. This effect was mediated by vagal adrenergic fibres originating from the cervical sympathetic ganglia probably acting via a β -adrenoceptor mechanism (Ahlman *et al.* 1978a). Ahlman *et al.* 1978b, Lundberg *et al.* 1978a). Also, bundles of unmyelinated nerve processes including catecholaminergic (CA) fibres were observed electronmicroscopically near the basement membrane of all examined EC in the guinea pig duodenum (Lundberg *et al.* 1978b). In the rat (Ohsumi *et al.* 1974) and the dog (Ahlman *et al.* 1978a) a vagal adrenergic innervation from the cervical ganglia to the gastrointestinal tract has also been observed by fluorescence histochemical studies.

The aim of the present investigation was to study the receptor mechanism involved in the release of 5-HT from EC *in vitro*.

Material and methods

II Sprague-Dawley rat (males, 250 g) were killed by decapitation. The proximal 5 cm of duodenum was dissected free and placed in ice-cold Krebs solution. The gut was opened to evert the mucosa, and then cut into 5 × 5 mm large pieces for further incubation.

The specimens were incubated for 30 min at 37°C in an oxygenated (5% CO₂, 95% O₂) Krebs solution containing the drug to be tested. In some experiments, where a receptor-blocking agent was to be tested, the specimen was preincubated with the blocker for 15 min before adding the agonist. The bath for further incubation (30 min). The controls in all experiments were incubated in Krebs solution for the same length of time.

Drug

The drugs tested were noradrenaline (NA) (L-arterenol bitartrate Sigma Chemical Comp.), adrenaline (A) (L-epinephrine bitartrate Sigma Chemical Comp.), isoprenaline (IP), (isoprenaline aspartate Apotolabogel), and pamine (DA) (3-hydroxy tyramine-HCl, Sigma Chemical Comp.) and acetylcholine (ACh) (acetylcholine-iodide BDH Chemical Limited) in the concentration range of 10⁻⁸–10⁻⁴ M. ACh was always combined with eserine (Physostigmine sulphate BDH Chemical Limited) 10⁻⁴ M. The blocking agent used were *D*-propranolol (Inderal IC1) 10⁻⁶ M and propranolol (Hjälte) 10⁻⁶ M, metoprolol (Sken® Hjälte) 2 × 10⁻⁶ M, phentolamine (Regitin® CIBA), 10⁻⁶ M, phenoxylbenzamine (PBA) (Dabem, Smith, Kline and French) 10⁻⁶ M. The ability of the blocking agents to antagonize the effect of A 10⁻⁶ M was tested.

III histochemistry and cytofluorimetry

After the incubation the gut specimens were immediately frozen in liquid propane, freeze-dried and treated with para-formaldehyde vapour for fluorescence microscopy according to the Hultarp-Falk technique. The cytofluorimetric method has been described in detail earlier (Ahlman *et al.* 1976a). From each specimen the 5-HT fluorescence intensity of 20 randomly chosen EC were recorded. As the relative measure of the 5-HT level of each EC the fluorescence intensity after 40 s of UV-illumination was recorded. (The reason for this is the rapid fading of the fluorescence, see Ahlman 1976). These values were expressed in arbitrary units, and the mean value of intracellular 5-HT in each control sample (the specimens incubated in drug-free Krebs solution) was set to 100 per cent. The mean value and SEM for each incubated specimen were calculated and compared with the control from the same animal. In experiments where the effect of different receptor antagonists were tested the specimens incubated with both the receptor-blocker and the agonist were compared with the specimens incubated with the agonist only. The results were statistically analysed by Student's *t*-test.

All specimens from one animal were processed simultaneously and all measurements were performed by the same person under identical conditions, using coded samples.

TABLE I. The effect of NA, A, IP, DA and ACh incubation on 5-HT content of EC. Agonist concentrations expressed in M. Mean values and S.E. of 20 cells from each specimen. Differences from Krebs incubated control indicated by * ($p < 0.01$) and ** ($p < 0.005$).

Agonist	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	M
NA	100 ± 5.2 96 ± 6.1 94 ± 7.0		$94 \pm 4.3^*$ $75 \pm 5.6^{**}$ $80 \pm 4.1^{**}$		$35 \pm 2.4^{**}$ $75 \pm 3.7^*$ $68 \pm 5.1^{**}$	NA
A	$76 \pm 3.9^*$ $80 \pm 4.3^*$ $81 \pm 3.9^{**}$	75 ± 4.4 80 ± 4.3 $82 \pm 4.3^*$	69 ± 4.1 $63 \pm 3.2^*$ $57 \pm 3.8^{**}$	$44 \pm 2.2^{**}$ $54 \pm 3.8^*$ 60 ± 3.4	$35 \pm 3.2^*$ $46 \pm 2.3^{**}$ 46 ± 2.4	A
IP	$73 \pm 3.4^{**}$ $57 \pm 3.7^{**}$ $75 \pm 3.6^{**}$		$75 \pm 3.3^{**}$ $58 \pm 3.8^{**}$ $66 \pm 4.0^{**}$		$88 \pm 4.3^*$ $38 \pm 4.5^*$ $68 \pm 3.5^{**}$	IP
DA	102 ± 6.4 96 ± 5.3 104 ± 7.6				98 ± 9.1 102 ± 7.3	DA
ACh	$85 \pm 6.9^*$ 124 ± 5.6 93 ± 4.4		83 ± 7.1 108 ± 5.6 $84 \pm 4.0^{**}$		88 ± 7.3 $86 \pm 3.4^{**}$ 86 ± 4.1	ACh

Results

The control specimens incubated in drug-free Krebs solution all showed a normal morphology with well visible green fluorescent nerve terminals and strongly yellow fluorescent EC.

The effect of receptor agonists

A distinct decrease of 5-HT fluorescence as compared to Krebs incubated controls was noted in the EC after incubation with NA, A and IP (Table I, Fig. 1). No difference between the three amines was noted except that A and IP were effective in the lowest concentration studied (10^{-6} M). The decrease in 5-HT fluorescence was concentration-dependent with NA and A (Fig. 1), while IP induced a maximal decrease already at 10^{-6} M!

DA did not affect the 5-HT content of EC in the doses tested (Table I). ACh in combination with eserine caused a small release of 5-HT from EC but was clearly less potent than the adrenergic substances (Table I).

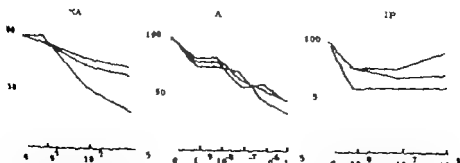


Fig. 1. The relationship between NA, A- and IP-concentration in the incubation medium and the release of 5-HT from the EC. Agonist concentrations expressed in M. For agonist names and S.E. see Table I.

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Fluorescence and cytofluorimetry

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All specimens from one animal were processed simultaneously and all measurements were performed by the same person under identical conditions, using coded samples.

se mechanism (Notal 1977). In all these instances the adrenergic release is probably one of several control mechanisms. The 5-HT release by adrenergic mechanisms may be part of the response caused by a general activation of the sympathetic system in a stress situation. The ultrastructural finding of several types of nerve processes near the basement surface of the EC indicate that the nervous control of these cells is not only an adrenergic mechanism but far more complex (Lundberg *et al* 1978 b).

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TABLE II The effect of adrenergic blocking agents on A induced 5-HT release from EC. Agonist antagonist concentrations expressed in M. Mean value and S.E. of 20 cells from each specimen. Difference from Krebs incubated controls * $p < 0.005$. Difference from A (10^{-7} M) incubated specimens, $p < 0.01$ and $p < 0.005$

Krebs	A 10^{-7}	A 10^{-7} d,l prop 10^{-6}	A 10^{-7} d-prop 10^{-6}	A 10^{-7} Metopr 10^{-6}	A 10^{-7} Phenitol- amin 10^{-4}	A 10^{-7} PHA 10^{-5}
100 \pm 4.7	64 \pm 3.5 ^o	73 \pm 3.9	55 \pm 3.4	55 \pm 4.2	59 \pm 2.6	49 \pm 1.7 ^o
100 \pm 4.3	67 \pm 4.1 ^o	100 \pm 5.5	73 \pm 4.2	60 \pm 2.4	62 \pm 6.0	53 \pm 3.0 ^o
100 \pm 5.0	57 \pm 2.8 ^o	69 \pm 3.8	55 \pm 4.4	40 \pm 3.1	44 \pm 2.0 ^o	

2. The effect of adrenergic blocking agents

The ability of different adrenergic blocking agents to antagonize the release of 5-HT from EC caused by A (10^{-7} M) was tested (Table II). d,l propranolol a β -adrenoceptor blocker agent was the only substance which had a significant blocking effect. The other antagonists tested had no blocking effect on the 5-HT release in the concentration tested.

Discussion

The *in vivo* incubation technique used in this study has shown to be a useful tool for studying the 5-HT release from EC, caused by various receptor agonists. A careful handling of the biopsies during the incubation procedure was of great importance, but at this point we considered the effects of agonist incubation were very reproducible.

Earlier cytofluorimetric investigations indicated the presence of an adrenergic receptor on the EC in the cat small intestine, possibly on the β -type (Ahlman *et al.* 1976 b). The results of the present experiments on the *in vivo* effects of adrenergic agonists and antagonists on the rat duodenal EC confirm this observation. The effect was dose-dependent for NA and A. d,l propranolol, a β -adrenoceptor blocking agent had a blocking effect on the 5-HT release caused by A. This blocking effect was in all probability not due to a membrane stabilization, since d propranolol, having a membrane stabilizing effect only did not block the 5-HT release. Equipotent concentrations of metoprolol, a selective β_1 -adrenoceptor blocker had no blocking effect. The results support the view that the adrenergic release of 5-HT from EC in rat was mediated via a true β -adrenoceptor mechanism, possibly of the β_2 -type. Cholinergic receptors may also be involved, since ACh (10^{-4} M) had a small but significant effect on the 5-HT content of the EC (Table I).

It is possible, however that metoprolol, phenitolamine, and PHA may have had an effect of their own in decreasing the 5-HT of the EC since the EC of specimens incubated with these blockers and A had a significantly lower 5-HT fluorescence intensity than incubated with A alone (Table II). A β -adrenergic release of hormones from endocrine cells has previously been suggested for the vagally induced release of gastrin in dog and man (Solich 1975 Christensen and Stadil 1976), for the adrenergic release of insulin from pancreatic islet cells (Lundquist 1971) and for the thyroxine secretion from the thyroid (Melander 1971). Another 5-HT-containing cell, the mast-cell has also been shown to have a β -ad

Differentiation between pre- and postjunctional α -receptors in guinea pig ileum and rabbit aorta

By

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Abstract

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The stimulation of proximal and terminal guinea pig ileum induced contractions which are mediated stimulation of cholinergic neurons. After blocking β -receptors, prejunctional α -receptors mediating release could be studied in these neurons. In proximal ileum the order of potencies of the following agents was clonidine (8), epinephrine (3), oxymetazoline (2), norepinephrine (1), phenylephrine (0.2). The intrinsic activities of clonidine and phenylephrine were not essential. In electrically stimulated small ileum practically identical results are obtained on the prejunctional α -receptor except that norepinephrine was ineffective, the drug instead acted as competitive blocker against norepinephrine. In blocking cholinergic receptors, i.e. nicotinic excitatory α -receptors located postjunctionally in the smooth muscle cells of terminal ileum could be studied. Under these conditions the order of potencies of the agents was oxymetazoline (40), epinephrine (7), phenylephrine (4), norepinephrine (1), clonidine (0.2). In contracting postjunctional α -receptors in rabbit aorta the order of potencies was oxymetazoline (1), norepinephrine (1), epinephrine (0.7), phenylephrine (0.2), clonidine (0.04). On the postjunctional receptor in terminal guinea pig ileum phenylephrine was 11 fold more effective in blocking the response of norepinephrine than was oxymetazoline. The response on the prejunctional receptor of the same preparation, phenylephrine (4 $\cdot 10^{-4}$ M) is ineffective on the prejunctional α -receptor of the cholinergic neuron. This blocked the postjunctional receptor of smooth muscle in guinea pig terminal ileum norepinephrine (1 $\cdot 10^{-4}$ M) suggested that the differences in the activities of clonidine and phenylephrine between prejunctional α -receptors located to cholinergic neurons and postjunctional α -receptors located to smooth muscle cells are due to pharmacological differences between the receptors. The results obtained in this study support this suggestion.

In an earlier paper (Wikberg 1977) results were presented which indicated that catecholamines induced relaxation of longitudinal smooth muscle of proximal guinea pig ileum by stimulation of α -receptors located to cholinergic neurons. The smooth muscle cells of this preparation lacked α -receptors although relaxatory β -receptors were present.

In two preliminary papers (Wikberg 1975, Wikberg *et al.* 1975) it was reported that the prejunctional α -receptor located to the cholinergic neurons of guinea pig small intestine showed a pharmacological difference in comparison to postjunctional α -receptors located to smooth muscle cells of guinea pig terminal ileum and rabbit aorta. Some of these findings

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use of non linear least square regression analysis (Parker and Ward 1971). The function is symmetrical, and curve has plotted as semi-logarithmic diagram. In the formula E is the effect, (A) the concentration of agonist, K the ED_{50} value, M the maximal effect and P a constant which is dependent on the shape of the curve. Intrinsic activity (α) has been defined

$$\alpha = \frac{M_{\text{tested substance}}}{M_{\text{acetylcholine}}}$$

$$\alpha = \frac{K_{\text{acetylcholine}}}{K_{\text{tested substance}}}$$

$$pD_2 = \log_{10} K$$

ratios (DR_1) were estimated from the K_1 of dose response curves in the absence and presence of antagonist

$$DR_1 = K_2/K_1$$

K_1 the ED_{50} of acetylcholine in the absence and K_2 in the presence of blocker. The dose ratios plotted by the method of Aronow and Schild (1959). According to classical receptor theory plot $\log_{10}(B)$, (where B is the concentration of the blocker), to $\log_{10}(DR_1)$ should give a straight line with slope of unity. The intercept of this line on the $\log_{10}(B)$ axis gives the pA_2 for the blocking drug. In calculating the data as fitted into straight line by linear least square regression and the 95% confidence interval of the slope was estimated (Droop and Smith 1966). The pA_2 from the experiment given by the intercept of unity slope linear regression of $\log_{10}(B)$ to $\log_{10}(DR_1)$ is the $\log_{10}(B)$ viz:

$$pA_2 = \left(\frac{\sum \log_{10}(B)_i - \sum \log_{10}(DR_1)_i}{n} \right) /$$

the apparent dissociation constant.

$$K_2 = \exp(-pA_2)$$

Statistics were calculated by analysis of variance and Student's t -test. Values in brackets after sensitivity determined values represent 95% confidence interval. Calculations were based on data stored on a Hewlett Packard 9830 calculator and the results were processed graphically on a 600 A plotter.

Following drugs were used. Atropine sulphate (ACO), cistidine (S.K. & F. Labs), cloazepam hydrochloride (Sandoz), dibenzylamine hydrochloride (Ayerst), dopamine hydrochloride (Sigma), 1-epinephrine (Sigma), isopropilol (Leo), isoprenaline chloride (ACO), methyln hydroxymethyl (Sandoz), norepinephrine bitartrate (Sigma), oxymetazoline chloride (Draco), phenylephrine (S.K. & F. Labs), tolazepam methylsulphate (Ciba), 1-phenylephrine hydrochloride (Sigma), procaine hydrochloride (Ciba), tetraethyl ammonium bromide (Mead-Johnson) tetrodotoxin (Sigma), tolazepam hydrochloride (Ciba).

Results

Guinea pig proximal ileum

Electric field stimulation of the guinea pig proximal ileum (coaxial stimulation) by 1 ms pulses at 0.1 Hz induced a twitch on each electric pulse. The twitches were completely blocked by tetrodotoxin (3×10^{-6} M) or atropine (3.5×10^{-6} M). A contraction elicited by histamine (7×10^{-4} M) was not at all affected by either tetrodotoxin or atropine (Fig. 1A, B).

have recently been confirmed by Drew (1977 b). It has been suggested that a pharmacological difference exists between presynaptic α -receptors located to adrenergic nerves and postsynaptic α receptors located in smooth muscle of several adrenergically innervated organs (Starke *et al* 1974 1975 Langer 1974 Dubocovich and Langer 1974 Sandoz and Nelson 1975 Drew 1977 a).

The present study was undertaken with the intention to investigate whether a pharmacological difference between α -receptors located to cholinergic neurons and those located to smooth muscle cells was present or not. α Receptors located to cholinergic neurons were studied in the electrically stimulated guinea pig proximal ileum (Paton 1955). α -Receptors located to smooth muscle cells were studied in rabbit aorta. In order to eliminate the possibility that a difference between the α -receptors was due to either species or organ differences, experiments were performed on the guinea pig terminal ileum as well. During electric stimulation α -receptors located to cholinergic neurons could be studied in this organ. After atropinization (Munroe 1951 1952) α -receptors located to smooth muscle cells could be studied in the same preparation.

Methods

Male guinea pigs or rabbits of either sex were killed by a blow on the head and bled. The smooth muscle organs were quickly removed, dissected free from adjacent tissue and mounted in organ bath containing Krebs solution as described previously (Wikberg 1977). The Krebs solution always contained atropine ($5 \cdot 10^{-6}$ M) in order to block β -receptors. The following preparations were used.

Guinea pig proximal ileum. Coaxial stimulation of the guinea pig ileum was performed essentially as described by Paton (1955). A piece of the ileum was removed approximately 10 cm from the ileocecal sphincter. 2 cm long pieces were prepared and mounted on a silver wire which was also inserted into the lumen of the preparation serving as an electrode. The silver wire was insulated when running over the lumen of the intestine and was connected with Grass FT 03 isometric transducer which permitted recording of the longitudinal muscle activity on a Grass polygraph. A second silver electrode, placed outside the lumen, in contact with the Krebs solution. At the lower end of the intestine the lumen was left open in order to avoid pressure differences between the outside and the inside of the preparation. The preparation was given an initial load of $1 \cdot 10^6$ dyne and left for 1 h before the experiment was started. Stimulation was performed with Grass SD9 stimulator and a home-constructed timer. Supramaximal current with 1 ms rectangular pulses of frequency of 0.1 Hz was used. The luminal electrode served as anode.

Guinea pig terminal ileum. 3 or 4 pieces of 1.5–2 cm length were removed from the terminal part of the ileum, starting where the small intestine joined colon. The preparation was mounted and stimulated electrically in the same manner as described for proximal guinea pig ileum above. The effects of agonists were first tested on the electrically stimulated preparation. Thereafter stimulation was stopped and the preparation was incubated with tropine $3.5 \cdot 10^{-6}$ M for at least 30 min. The agonists were then tested again on the same preparation.

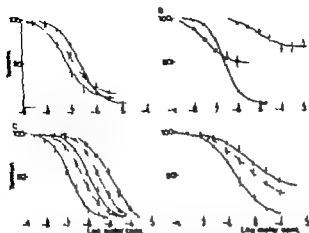
Rabbit thoracic aorta was removed and cut helically into 4–20 mm strips and mounted in the organ bath. The tension was recorded isometrically. An initial tension of $2 \cdot 10^6$ dyne was applied to the preparation 1 h before the experiment was started.

In all experiments when antagonists were tested, the preparations were incubated for at least 30 min with the drug before any experiments were performed.

Statistical methods

Dose response curves were treated statistically. Each dose response curve was fitted into a logistic function

$$E = M \frac{[A]^P}{[A]^P + K^P}$$



3 Dose response curves by adrenergic agonists in the coarsely stimulated guinea pig uterine smooth muscle. Continuous sigmoidal lines represent the regression of the logistic function to the experimental data. Epinephrine — \square , tolazoline Δ — Δ , norepinephrine \bullet — \bullet (n = 7). B Clonidine \circ — \circ , norepinephrine \bullet — \bullet , phenylephrine — \square (n = 5). C Test of tolazoline in the coarsely stimulated guinea pig uterine smooth muscle. Control response by norepinephrine alone \bullet — \bullet , tolazoline 10^{-6} M — \square , tolazoline 3×10^{-6} M Δ — Δ , tolazoline 10^{-5} M \circ — \circ , tolazoline 3×10^{-5} M \square — \square (n = 4-5). D Test of phentolamine. Control response of norepinephrine alone \bullet — \bullet , phentolamine 10^{-6} M — \square , phentolamine 2.7×10^{-6} M Δ — Δ (n = 6).

10^{-6} M), klemastin (3×10^{-6} M), thioridazine (4×10^{-6} M) and haloperidol (2×10^{-6} M) most completely inhibited the response to electric stimulation. Evaluation of these substances on eventual blocking abilities on the effect of norepinephrine in the electrically stimulated uterus was therefore impossible.

TABLE I. Estimates of pD_2 , relative affinity and maximum activity of adrenergic agonists in atropinized guinea pig uterine smooth muscle, coarsely stimulated guinea pig uterine smooth muscle and slices and rabbit aorta

Trimmed uterus - Atropine 3.5×10^{-6} M				Coarsely stimulated		
Drug	pD_2	rel. aff.		pD_2	rel. aff.	I.A.
Nor-epinephrine	34.379 (3.47-4.11)	1	1	6.67 (6.46-6.88)	1	1
Epinephrine	9.471 (3.96-5.48)	7	0.96 (0.82-1.10)	7.06 (6.48-7.65)	3	0.96 (0.92-1.01)
Phenylephrine	9.464 (4.36-4.91)	4	0.62 (0.37-0.86)	—	—	0 (see text)
Tolazoline	6.625 (5.66-6.45)	40	0.19 (0.08-0.30)	7.32 (7.00-7.63)	3	1.08 (0.99-1.16)
Clonidine	8.378 (3.45-4.11)	0.2	0.22 (0.12-0.33)	7.68 (7.33-7.98)	20	0.66 (0.41-0.91)
Dopamine	7.531 (3.28-3.74)	0.07	0.40 (0.23-0.57)	5.34 (5.22-5.46)	0.09	0.77 (0.64-0.91)

Rabbit aorta				Guinea pig uterus, coarsely stimulated		
Drug	pD_2	rel. aff.	I.A.	pD_2	rel. aff.	I.A.
Nor-epinephrine	15.647 (6.36-6.58)	1	1	11.633 (6.57-6.70)	1	1
Epinephrine	8.644 (6.24-6.66)	0.7	1.09 (0.74-1.43)	7.706 (6.58-7.54)	3	0.99 (0.95-1.04)
Phenylephrine	7.539 (3.31-6.87)	0.2	1.11 (0.55-1.67)	8.500 (4.68-5.33)	0.02	0.38 (0.22-0.54)
Tolazoline	7.795 (6.93-7.18)	3	1.12 (0.75-1.50)	7.626 (6.49-7.23)	2	0.95 (0.87-1.03)
Clonidine	8.519 (3.03-5.34)	0.04	0.13 (0.10-0.17)	8.751 (7.05-7.97)	8	0.52 (0.37-0.66)

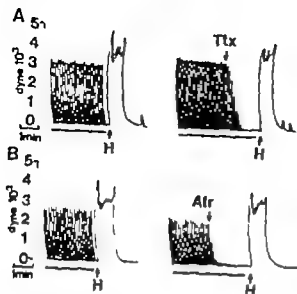


Fig. 1 Responses of preparation from guinea pig proximal ileum. Coaxial stimulation 1 ms pulses at 0.1 Hz indicated by horizontal bars. A At arrows histamine 7×10^{-6} M and tetrodotoxin 3×10^{-6} M (Ttx) B At arrows histamine 7×10^{-6} M (H) and tetrodotoxin 3×10^{-6} M (Ttx). Washing indicated by open dots.

In the experiments to be described sotalol (5×10^{-6} M) was always present in order to block β -receptors (Wikberg 1977).

Tests of agonists on the guinea pig proximal ileum. Typical responses of the ileum tested on the coaxially stimulated guinea pig proximal ileum are shown in Fig. 1. Cumulative dose response curves of these drugs are shown in Fig. 3 A B and the results are summarized in Table 1. Both epinephrine and oxymetazoline were slightly more potent than norepinephrine. The maximal inhibitory effect obtained by these drugs was not significantly different from that of norepinephrine. The intrinsic activity of phenylephrine was significantly lower (0.38 (0.22–0.54) $p < 0.001$) and its relative affinity only 0.52 (0.37–0.68) $p < 0.001$. Clonidine also showed submaximal intrinsic activity in comparison with norepinephrine (0.52 (0.37–0.68) $p < 0.001$). Despite its low intrinsic activity clonidine was slightly more potent than norepinephrine.

Test of antagonists on the guinea pig proximal ileum. The displacement of dose-response curves of norepinephrine by several drugs was investigated for the evaluation of their blocking properties in the coaxially stimulated guinea pig ileum. Several of the drugs that were tested were found to depress the electric twitch response markedly. Thus, promethazine

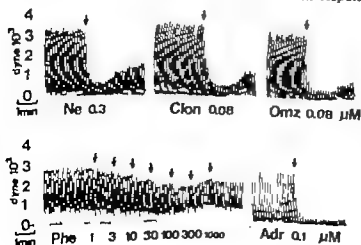
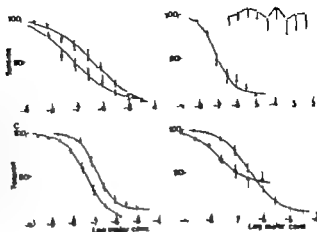


Fig. 2 Responses of preparation from guinea pig proximal ileum stimulated coaxially by 1 ms pulses at 0.1 Hz. Arrows indicate the addition of norepinephrine 3×10^{-6} M (Ne), clonidine 8×10^{-6} M (Clon), oxymetazoline 8×10^{-6} M (Omz), phenylephrine 1×10^{-4} M (Phe) and epinephrine 1×10^{-4} M (Adr).

4 Dose response curves of
sergic agonists in the cox-
stimulated terminal ileum.
Epinephrine — ●—●—
Norepinephrine — ○—○—
Phenylephrine — □—□—
Oxymetazoline — △—△—
Dopamine — ◇—◇—
Clovidine — ⊙—⊙—
Epinephrine — ⊕—⊕—



Test of cimetidine on the guinea pig proximal ileum Experiments were performed to estimate if cimetidine ($3 \cdot 10^{-6}$ to 10^{-5} M) influenced the response of oxymetazoline (10^{-6} M) and clovidine ($1 \cdot 10^{-6}$ M) in electrically stimulated guinea pig proximal ileum. Cimetidine had no blocking effect on the responses to the imidazolines (Table II A). There was possibly a significant potentiation of the effect of oxymetazoline by cimetidine (10^{-6} M) at $p < 0.05$, however.

Guinea pig terminal ileum

The terminal part of the guinea pig ileum responded to electric stimulation in a similar way as the proximal ileum. The electric stimulation was blocked by tetrodotoxin ($3 \cdot 10^{-7}$ M) or atropine ($3 \cdot 5 \cdot 10^{-6}$ M) while the response to histamine ($7 \cdot 10^{-7}$ M) was not affected at all.

Test of agonists in the terminal ileum, inhibitory response Norepinephrine, epinephrine, oxymetazoline, clovidine and dopamine depressed the twitch response to coaxial stimulation in the guinea pig terminal ileum in a similar way as was observed in guinea pig proximal ileum. Phenylephrine did not inhibit the contraction, however, but instead often potentiated the twitch response to electric stimulation.

Competitive dose response curves obtained on the coaxially stimulated terminal ileum, by these drugs are given as Fig. 4 and 6 A and the results are summarized in Table I A. Epinephrine and oxymetazoline were slightly more potent than norepinephrine. The intrinsic activity of epinephrine and oxymetazoline did not significantly differ from that of norepinephrine. Clovidine was about 20 times more potent than norepinephrine but its intrinsic activity was less (0.66 (0.41–0.91)). The relative affinity of dopamine was 0.09 and its intrinsic activity was submaximal (0.77 (0.64–0.91)). Phenylephrine did not cause any dose related inhibition of the electrical stimulation. In three of the preparations there was an inhibition of the electric contraction to maximally about 75%. The rest of the preparations showed potentiation in all the concentrations tested (< 4) or slight inhibition in the lower and potentiation in the higher concentrations of phenylephrine. The variability of the responses did not allow any determination of the drug parameters.

TABLE II A. Effect of clometidine on the inhibitory responses of oxymetazoline and clonidine on the stimulated guinea pig ileum. The effect of the agonists are given as the percentage inhibition of the twitch response. \pm S.E.M. (n.s. = not significant)

Agonist	Clometidine			
	Control	$3 \cdot 10^{-6}$ M	$1 \cdot 10^{-5}$ M	$3 \cdot 10^{-5}$ M
Oxymetazoline ($1 \cdot 10^{-6}$ M) (n = 5)	36.9 ± 2.9	67.1 ± 4.4 p < 0.05	57.3 ± 4.3 n.s.	57.0 ± 5.5 n.s.
Clonidine ($1 \cdot 10^{-6}$ M) (n = 6)	46.7 ± 5.3	53.5 ± 6.4 n.s.	46.4 ± 4.5 n.s.	35.9 ± 4.4 n.s.

TABLE II B. Effect of tolazoline on the inhibitory responses of epinephrine, phenylephrine, oxymetazoline and clonidine on the electrically stimulated ileum. The effect of the agonists are presented in Table II A

Agonist	Tolazoline	
	Control	$1 \cdot 10^{-6}$ M
Epinephrine ($1 \cdot 10^{-6}$ M) (n = 4)	91.1 ± 0.8	16.3 ± 9.3 p < 0.01
Phenylephrine ($1 \cdot 10^{-6}$ M) (n = 4)	32.8 ± 3.4	18.9 ± 1.2 p < 0.01
Oxymetazoline ($1 \cdot 10^{-6}$ M) (n = 4)	64.9 ± 4.3	19.6 ± 1.9 p < 0.001
Clonidine ($1 \cdot 10^{-6}$ M) (n = 4)	50.5 ± 6.7	25.3 ± 2.9 p < 0.01

Tolazoline ($1 \cdot 10^{-6}$ – $3 \cdot 10^{-5}$ M) caused a parallel displacement of the dose response curves of norepinephrine to the right (Fig. 3 C). Analysis of variance showed that there were neither any significant effects on the initial twitch contraction (p > 0.25), nor on the final maximal response caused by norepinephrine (p > 0.25) induced by tolazoline. Neither were there any effects on the slopes of the dose response curves (p > 0.1). The slope of the linear regression of log (B) to log (DR 1) (n = 19 from 4 expts.) was not significantly different from unity (0.96 (0.74–1.19)). The pA_1 calculated from the expts. was 6.09 (6.09–6.71) and the apparent K_D $4.0 \cdot 10^{-6}$ M.

Phentolamine $8.0 \cdot 10^{-6}$ M and $2.7 \cdot 10^{-5}$ M depressed the twitch response by electric stimulation to 75.3 (65.6–85.0)% and 52.2 (49.2–65.2)% respectively. The dose response curve for norepinephrine was not displaced in a typically parallel manner by phentolamine but the maximal inhibitory effect of norepinephrine was diminished (Fig. 3 D). The slope of the linear regression of log (B) to log₁₀ (DR 1) was far different from unity (−0.88–+1.33) and there was no significant correlation at all in the regression (p > 0.25). The pA_1 calculated from the data was 5.96 (5.61–6.31).

The blocking properties of tolazoline on the responses of epinephrine, phenylephrine, oxymetazoline and clonidine were also investigated in the guinea pig proximal ileum. Tolazoline ($1 \cdot 10^{-6}$ M) counteracted the effect of epinephrine ($1 \cdot 10^{-6}$ M), phenylephrine ($1 \cdot 10^{-6}$ M), oxymetazoline ($1 \cdot 10^{-6}$ M) and clonidine ($1 \cdot 10^{-6}$ M) (Table II B).

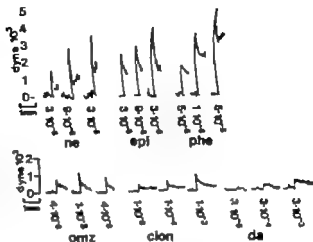


Fig. 1 Responses of adrenergic agents in atropinized guinea pig terminal ileum. Responses to norepinephrine: 3×10^{-8} to 3×10^{-5} M; epinephrine: 3×10^{-8} to 3×10^{-5} M; phenylephrine: 3×10^{-8} to 3×10^{-5} M; ornithine: 3×10^{-8} to 3×10^{-5} M; clonidine: 3×10^{-8} to 3×10^{-5} M; dantrolene: 3×10^{-8} to 3×10^{-5} M.

the slope of the curve ($p = 0.1$) or on the maximal response ($p = 0.8$) (Fig. 5 B). The pA_{50} of norepinephrine was 5.39 (4.51–6.27) and K_D 4.1×10^{-6} M. Norepinephrine (3×10^{-6} M) depressed the electric stimulation to 80.6 (72.4–88.8)%. The addition of norepinephrine caused a displacement of the dose response curve of norepinephrine to the right without affecting the maximal inhibition induced by norepinephrine (Fig. 5 C). Phenoxymethamine (4×10^{-6} M) slightly depressed the electric stimulation of terminal ileum. The electric contraction after phenoxymethamine was 89.1 (65.1–113.0)% of the control. The addition of phenoxymethamine did not seem to have any blocking properties on the effect of norepinephrine in this concentration (Fig. 5 D). Since phenylephrine showed very little agonistic activity on the electric contractions of the electrically stimulated terminal ileum it was of interest to investigate if the drug possessed blocking properties against norepinephrine in this preparation. Phenylephrine (5×10^{-6} M) caused a clear displacement of the dose response curve of norepinephrine to the right without affecting the slopes ($p = 0.8$) or the maximal response ($p = 0.8$). Neither did phenylephrine affect the contraction following electric

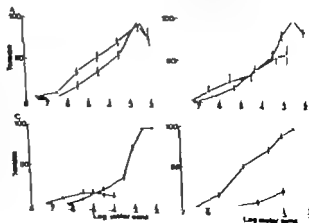
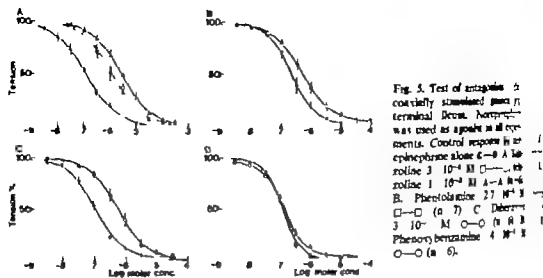


Fig. 2 Dose response curves of adrenergic agents in atropinized guinea pig terminal ileum. A. Norepinephrine (—●—) and phenylephrine (---●---) (A 5). B. Norepinephrine (—●—) and phenoxymethamine (---●---) (A 5). C. Ornithine (—●—) and clonidine (---●---) (A 5). D. Norepinephrine (—●—) and dantrolene (---●---) (A 5).



Test of antagonists in the terminal ileum, inhibition of inhibitory response Tolazoline ($3 \times 10^{-4} - 1 \times 10^{-3}$ M) shifted the dose response curve of norepinephrine to the right (Fig. 5 A). Tolazoline did not significantly affect the initial twitch response by electric stimulation ($p > 0.25$), the maximal inhibition induced by norepinephrine ($p > 0.25$) or the slope of the dose response curves ($p > 0.25$). The slope of the linear regression of the Schild plot was not significantly different from unity (0.73 (0.13-1.33)) ($n=12$ from 6 experiments). The pA of tolazoline was 6.38 (6.14-6.61) and the apparent K_0 4.2×10^{-4} M. Phentolamine (2.7×10^{-4} M) depressed the electric stimulation of the guinea pig terminal ileum to 79% (68.5-89.5) of the initial contraction. The drug caused a slight shift of the dose response curve of norepinephrine to the right without causing any significant effect.

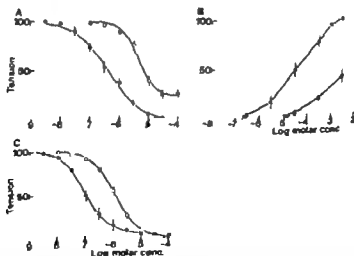


Fig. 6 A. Test of adrenergic agonists on coxially stimulated guinea pig terminal ileum. Norepinephrine 5×10^{-6} M. $\bullet-\bullet$ dopamine 1×10^{-5} M. $\circ-\circ$ (n=7). B. Tests on guinea pig terminal ileum pretreated with tropic acid 2.5×10^{-3} M. Norepinephrine 5×10^{-6} M. $\bullet-\bullet$ dopamine 1×10^{-5} M. $\circ-\circ$ (n=7). C. Demonstration of the blocking properties of phentolamine on the response of norepinephrine in the coxially stimulated guinea pig terminal ileum. Control response by norepinephrine 5×10^{-6} M. $\bullet-\bullet$ response of norepinephrine after 15 min pretreatment with phentolamine 5×10^{-4} M. $\square-\square$ (n=4).

oxybenzamine (4×10^{-6} M) depressed the maximal response to norepinephrine 9 C). Debenzamine (3×10^{-6} – 3×10^{-5} M) caused a similar depression of the maximal response obtained by norepinephrine as well as phenoxybenzamine (Fig. 9 D).

α-1a receptors

Effect of agonists in the rabbit aorta. The α -receptor agonists tested in the guinea pig ileum and terminal ileum were for comparative purposes tested in the rabbit aorta. The results are summarized in Table I B. The maximal effects obtained by norepinephrine, epinephrine, phenylephrine and oxymetazoline did not differ significantly. Oxymetazoline was slightly more potent while epinephrine and phenylephrine were slightly less potent than norepinephrine. The effect of oxymetazoline was slow at the onset and extremely persistent and did not disappear after 3 h of washing. The contractile effect of clonidine was very low (a. 0.13–0.017) and its relative affinity only 0.04. The contractions induced by clonidine were much weaker than those of norepinephrine and resembled those of oxymetazoline although they were also weaker. The effect of clonidine in the dose range 1×10^{-6} – 1×10^{-5} M was blocked by phentolamine 2.6×10^{-6} M.

Test of antagonists in the rabbit aorta. Phentolamine 8.0×10^{-6} – 8.0×10^{-5} M caused a parallel displacement of the dose response curve of norepinephrine to the right without affecting the slope ($p > 0.1$) or the maximal response ($p > 0.25$). The slope of the Schild plot for phentolamine was not significantly different from unity (1.01 (0.81–1.21)) ($n = 15$ from 6 expts.). The pA calculated from the experiment was 7.59 (7.18–7.81) and K_B 7.4×10^{-6} M. Tolazoline 3×10^{-6} – 3×10^{-5} M caused a displacement of the dose response curves of norepinephrine to the right without affecting the maximal responses ($p > 0.25$). There was no significant effect on the slopes of the dose response curves by tolazoline ($p < 0.01$) because the slopes became steeper. In spite of this the slope of the Schild plot was close to unity (0.98 (0.78–1.17)) ($n = 18$ from 6 expts.). The pA of tolazoline was 5.51 (5.6–5.85) and the apparent K_B 3.1×10^{-6} M.

Discussion

In the present work two lines were used in the attempts to characterize α -receptors present in the studied organs. When agonists were tested, dose-response curves of the drugs were obtained and the relative affinity and intrinsic activity were taken as a primitive characterization of the drug-receptor interaction. When antagonists were tested, dose-response curves of norepinephrine were made in the absence and presence of blocker and the data was analyzed by the Schild equation (Arunlakshana and Schild 1959).

Alpha-receptors in cholinergic neurons

The results obtained on the electrically stimulated terminal ileum were almost identical with those obtained on proximal ileum, and they will therefore be discussed together.

Clonidine has been suggested to stimulate α -receptors in central and peripheral nervous tissues including the cholinergic neurons of the gastrointestinal tract (Kobinger and Walland 1972, Starks and Altman 1973, Bolms *et al.* 1974, Kobinger and Pachler 1975).

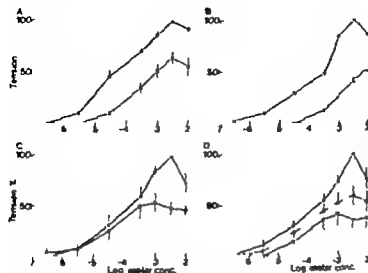


Fig. 9 Test of atropine antagonism on the terminal ileum contractile response. Norepinephrine was used as agonist in all experiments. Control response by atropine alone is shown in A. Table 1. 10^{-6} M \square — \square (a) 6.2. Phenylephrine $2.7 \cdot 10^{-6}$ M (a=7). C. Oxymetazoline 10^{-6} M \circ — \circ (a=8). D. Clonidine $3 \cdot 10^{-6}$ M \circ — \circ (a=6).

stimulation ($p < 0.6$) of the preparations (Fig. 6C). The pA of phenylephrine was estimated to be 4.20 (3.66–4.73), and K_D was estimated to be $6.3 \cdot 10^{-6}$ M.

Test of agonists on the terminal ileum contractile response In the presence of atropine ($3.5 \cdot 10^{-6}$ M) and sotalol ($5 \cdot 10^{-6}$ M) norepinephrine, epinephrine and phenylephrine induced strong contractions of the guinea pig terminal ileum. Under these conditions oxymetazoline ($4 \cdot 10^{-6}$ – $4 \cdot 10^{-5}$ M) clonidine ($1 \cdot 10^{-6}$ – $1 \cdot 10^{-5}$ M) and dopamine ($3 \cdot 10^{-6}$ – $3 \cdot 10^{-5}$ M) induced only weak contractions (Fig. 7).

Non-cumulative dose response curves of the agonists were obtained from tests on the terminal ileum and the results are shown in Fig. 8, and in Table 1. The dose response curves were often unsymmetric. At high concentrations of norepinephrine and epinephrine inhibition of the dose response curves was often observed. Due to the unsymmetrical shape of the dose response curves in the atropinized terminal ileum, conventional graphic methods were used in the analysis of the data instead of the logistic function model.

Epinephrine was slightly more potent than norepinephrine and its intrinsic activity did not significantly differ from that of norepinephrine. Phenylephrine was slightly more potent than norepinephrine but its intrinsic activity was less (0.62 (0.37–0.86)) ($p < 0.01$). Oxymetazoline was 40 times more potent than norepinephrine but despite of this its intrinsic activity was low (0.19 (0.08–0.30)) ($p < 0.01$). Due to the high concentration needed the maximal responses of dopamine and clonidine were probably not reached. The relative affinity of clonidine was estimated to be less than 0.2 and its intrinsic activity more than 0.22 (0.12–0.33). The relative affinity of dopamine was less than 0.07 and the intrinsic activity more than 0.40 (0.23–0.57).

Test of antagonists in the terminal ileum, inhibition of contractile response Phentolamine ($2.7 \cdot 10^{-6}$ M) shifted the dose response curve of norepinephrine parallelly to the right although the maximal effect of norepinephrine was not reached after the blocking drug (Fig. 9B). The pA of phentolamine calculated from the experiment was 7.13 (6.76–7.50) and the estimated dissociation constant $7.4 \cdot 10^{-7}$ M. Tolazoline ($1 \cdot 10^{-6}$ M) also caused a parallel shift to the right of the dose response curve of norepinephrine (Fig. 9A). The pA of tolazoline was 6.22 (5.83–6.60) and the estimated dissociation constant $6.1 \cdot 10^{-7}$ M.

not fulfilled. Sanders *et al.* (1975) suggested that tolazoline was a weak α -adrenergic antagonist in rabbit aorta. It is possible that a weak intrinsic activity of tolazoline might have been responsible for the observed increase in the slopes of the dose response curves of pinephrine.

partition between α -receptors in cholinergic neurons and smooth muscle cells

There was clear-cut evidence for the existence of subtypes of α -receptors in the studied preparations were derived from the tests with adrenergic agonists. There was a hundredfold difference between the relative affinities of clonidine on inhibition of electric stimulation of ileum and contracting the same organ after atropine. The difference between relative affinities of clonidine on electrically stimulated proximal ileum and rabbit aorta was of similar magnitude about 160 in relation to norepinephrine, phenylephrine being four times more potent in contracting the atropinized terminal ileum whereas it was not completely ineffective in inhibiting contractions elicited by electric field stimulation of the same organ. The drug actually was a competitive blocker to norepinephrine in this preparation, which indicated that phenylephrine had affinity but no activity at the α -receptor of the neurons. The difference between the relative affinities of phenylephrine on electrically stimulated ileum and rabbit aorta was approximately 10. The drug showed maximal intrinsic activity in rabbit aorta, whereas it was only a weak agonist in ileum. The other studied agonists, i.e. oxymetazoline, epinephrine and dopamine did not show any selective action on α -receptors located to neurons or smooth muscle cells.

Phenolamine was 55 fold more effective in blocking the α -receptor located in smooth muscle of guinea pig terminal ileum than in blocking the α -receptor of the cholinergic neuron. The blockade induced by phenolamine on the α -receptor of the neurons might not be competitive. The slope of the Schild plot obtained from proximal ileum was far from unity and phenolamine diminished the maximal effect induced by norepinephrine.

Tolazoline was 8 times more potent in blocking α -receptors in cholinergic neurons of guinea pig than in blocking α -receptors in the rabbit aorta. The potency of tolazoline to block α -receptors in cholinergic neuron and smooth muscle cells of guinea pig terminal ileum was of the same magnitude, however.

Phenylbenzamine induced non-competitive blockade of the α -receptor located to smooth muscle of guinea pig terminal ileum but it was ineffective on the receptor of the cholinergic neurons, even though the same concentration and incubation time of the blocker was used in both tests.

Nomenclature of α -receptors

Subtypes of α -receptors might be named by their anatomical localization such as pre- or postsynaptic. There are certain disadvantages inherent with this nomenclature, however. Postsynaptic α -receptors could be localized in nervous tissue such as sympathetic ganglia (De Groot and Völz 1966) as well as in effector organs. Until the pharmacological properties of such or other α -receptors are compared with α -receptors of adrenergic or cholinergic nerves it is preferable not to refer to these receptors by their localization. Langer (1974) suggested that presynaptic α -receptors of adrenergic neurons should be

Vizi 1976). Oxymetazoline has also been suggested to have α -stimulatory properties in rabbit intestine (Mujic and van Rossum 1965) and the rabbit aorta (Sanders *et al* 1971).

The experiments of the present study indicated that the tested agonists inhibited electrically stimulated ileum by stimulating α -receptors. The agonists induced dose-related effects after β -receptors had been blocked by sotalol. Tolazoline acted as a competitive blocker against norepinephrine. This conclusion was reached since all criteria for competitive antagonism were fulfilled. The effects of the other tested agonists were also blocked by tolazoline, indicating that all agonists acted through stimulating α -receptors. The blocking properties of dibenamine also supported the theory that the adrenergic neuron of the cholinergic neuron was an α -receptor.

Recently some evidence has been presented that clonidine is capable of stimulating H_2 -receptors (Karppanen and Westerman 1973, Csongrady and Kobayashi 1974, Andersson *et al* 1978). In this study there was no evidence that clonidine or other tested drugs activated H_2 -receptors in electrically stimulated proximal ileum since the responses to these drugs were not blocked by cimetidine. Dopamine was tenfold less potent than norepinephrine in the electrically stimulated terminal ileum. The receptor of the cholinergic neuron should therefore probably not be classified as dopaminergic.

Alpha-receptor in smooth muscle

Guinea-pig terminal ileum After blocking β -receptors with sotalol and cholinergic receptors with atropine the adrenergic agonists contracted the terminal ileum. Under these conditions it is probable that the responses were mediated through stimulation of receptors located to the smooth muscle cells.

It is probable that norepinephrine acted by stimulating α -receptors since both tolazoline and phentolamine caused parallel displacements of the dose response curve. The maximal effect of the agonist was not reached after the blockers. This may be explained by the presence of an inhibitory effect of norepinephrine at high concentrations. An inhibitory effect of catecholamines and related drugs, independent of α - or β -receptors, was present in denervated guinea pig proximal ileum when high concentrations were used (Waller 1977).

The blocking effect of phenoxybenzamine and dibenamine against norepinephrine indicates that the excitatory receptor in guinea pig proximal ileum should be classified as an α -receptor. The haloalkylamines induced a non-competitive type of receptor blockade since the dose response curves of the agonist were not at all shifted to the right.

Rabbit aorta The relative affinities of norepinephrine, epinephrine, phenylephrine and oxymetazoline on rabbit aorta found in this study were close to those reported by others in the same preparation (Bevan and Osher 1965, Furchtgott 1970, Sanders *et al* 1975). The intrinsic activity and relative affinities of clonidine on rabbit aorta was similar to that found by Starke *et al* (1974) on rabbit pulmonary artery. In rabbit aorta clonidine probably stimulated α -receptors since the weak contractions were blocked by phentolamine.

It is concluded that phentolamine acted as a competitive blocker against norepinephrine since all criteria for competitive antagonism were fulfilled. Tolazoline probably also blocked the effect of norepinephrine competitively although all criteria for competitive antagonism

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referred to as α_2 -receptors while postsynaptic α -receptors should be referred to as α_1 -receptors. This suggestion was also notified by Drew (1977a). According to this nomenclature it is suggested that α_2 -receptors are defined as those α -receptors for which the relative potency is clonidine > phenylephrine. Furthermore α_1 -receptors are defined as those for which the relative potency is phenylephrine > clonidine. By this definition α_2 -receptors were present in the smooth muscle of guinea pig terminal ileum and in rabbit aorta. α_1 -Receptors were present in the cholinergic neurons of proximal and terminal guinea pig ileum.

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Autoradiography on erythrokinesis and multihemoglobins in juvenile *Salmo salar* L. at various respiratory gas regimes

By

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Abstract

HÄRDIG J., L. Å. OLSSON and L. B. HÖGLUND: *Autoradiography on erythrokinesis and multihemoglobins in juvenile Salmo salar L. at various respiratory gas regimes.* Acta physiol. scand. 1978 103 240-251.

The kinetics of erythropoiesis in 5-6 month old *Salmo salar* L. was correlated to 4 combinations environment (PO_2 and pH/PCO_2). Given single doses of ^{59}Fe at the start of continuous flow between the incorporation was examined in circulating red blood cells (RBC) by autoradiography on blood smears. The proportions of immature labelled immature and labelled mature erythrocytes were calculated for samples taken at intervals up to 52 days. Compared to control fish kept at a PO_2 corresponding to 90% air saturation and pH/PCO_2 7.6/8 pH units, mmHg, oxygen depletion to 50% air saturation slowed the proliferation of RBC stem cells and enhanced RBC maturation. Certain parameters, restricted hypoxia at a PCO_2 in the respiratory water raised to 23 mmHg (pH 7.1-7.2), stimulated proliferation but did not affect the output of mature RBC. Simultaneously lowered PO_2 and raised PCO_2 to the levels mentioned above obviously stressed the fish, as the effect of lowered PO_2 per se was not manifested. Radioactive ^{59}Fe was traced to all electrophoretically separable protein fractions from RBC hemolysates. The relevance of blood physiological criteria for probing the fitness or well being of fish in environmental requirements conditions has been discussed.

An autoradiographic approach for studying erythrokinetical events in teleosts environmentally exposed to well defined environmental changes, does not seem to have been made previously. The present study was initiated in order to determine if the exposure for about 7 weeks to different respiratory gas regimes effects the "multihemoglobin pattern" (Koch 1977) and erythropoiesis in young salmon. On the whole, the present knowledge of the kinetics of fish erythropoiesis is relatively scanty. As in mammals the hemoglobin content and hematocrit is supposed to increase in the blood of fish due to environmental hypoxia (Phillips 1947, Chiba 1965, Randall 1970). At least in salmonids these blood parameters and the total number of erythrocytes also increase at rising water temperatures (Dewilde and Houston 1967, Miles and Smith 1968). Powers (1932) gave evidence in *Ictalurus punctatus* for an increased number of circulating red blood cells (RBC) in response to oxygen depletion combined with a slight increase in carbon dioxide tension in the respiratory water.

Injection of serum from anemic or hypoxic fish stimulates erythropoiesis in the blue smelt (*Trichostema trichopterus*) (Zanjan *et al.* 1969). In the same species Yu *et al.* (1969) demonstrated that injected radioactive iron is stored in the spleen, kidney and liver nodules, which is a supply of iron used when erythropoiesis is stimulated. Thus erythropoiesis in fish seems to be controlled by similar mechanisms as found in mammals (Yu *et al.* 1969).

Immature new circulating RBC appear as immature round erythroblasts which can be formed microscopically from more elongated mature erythrocytes (Topf 1955). Härdig (1969) has discussed this matter more closely along with studies on haemoglobin synthesis in young salmon (*Salmo salar*) based on observations of the incorporation of radioactive

attempt has been made in the present study to apply continuous flow bioassays (Härdig 1969, Börjeson and Hoglund 1976) in order to correlate the kinetics of RBC production and RBC maturation in juvenile Baltic salmon (*Salmo salar*) to four great combinations of PO_2 and PCO_2 in the respiratory water. At the start of the experiment for 52 days each specimen was injected intraperitoneally with a single dose of ^{59}Fe . The incorporation of radiotracer into immature and mature red cells has been studied on autoradiograms of blood smears from 5 to 7 specimens exposed to each treatment for various lengths of time (cf. p. 242).

Simultaneously the incorporation of radioactive iron into the electrophoretically separable fractions of RBC haemolysates was checked on fish from all test groups.

Material and methods

Fish. In all 192 live to six month old salmon parr (*Salmo salar* L.) with mean weight and S.D. of 2.3 g (range 2-9 g) were used in the tests. The normal material was supplied by the Fishery Board of Sweden from the hatchery and rearing plant at Åhlsby at the River Dälälven near its outlet to the Baltic. It represented a local population of Baltic salmon derived from eggs of many females fertilized by a few males. Before used in the experiments all fish were preacclimated for 2 weeks in our laboratory by running aerated Uppsala tap water equal to the control conditions in the assays. In parallel they were fed daily even during the tests with salmon starter fodder pellets (Astra-Eko, Mölndale, Sweden).

Water quality. The original feed water was Uppsala tap water. It is subsoil water from an entry flowing in some parts through areas with limestone deposits. It is poor in oxygen and has fairly constant ion composition: Ca^{2+} 2.0-2.2 mmol/l, Mg^{2+} 0.5-0.6 mmol/l, and HCO_3^- about 5 mmol/l are the prevailing constituents (Hoglund *et al.* 1974). About 30 fish were placed in each of 4 continuous flow test vessels. 5-7 specimens from each batch were removed at intervals and then killed. Those specimens were kept in the test troughs for at least 52 days. Test group I being the "control" was run equal to the acclimation environment in all oxygenated tap water (90 air saturation) at moderately high carbon dioxide tension (about 8 mmHg at pH 7.6). In the 3 other vessels the following combinations of respiratory gas parameters were established, lowered oxygen content and moderate carbon dioxide tension in Test group II, sufficient oxygen and raised PCO_2 in Test group III, lowered PO_2 as well as raised PCO_2 in Test group IV as shown in Table 1. PCO_2 , PO_2 and pH are maintained constant and controlled daily for the whole period of 52 days. Each test vessel containing 60 l and drained at the rate of 530-630 ml/min. A definite PO_2 was secured by the accurate mixing of non-aerated and air-saturated tap water. Carbon dioxide over-saturation was kept at the desired fixed PCO_2 by the continuous addition of the appropriate amounts of hydrochloric acid to the actual drainage of bicarbonate-rich supply water (Hoglund 1964, Hoglund and Härdig 1969). The oxygen content was determined by Winkler titration as modified by Carpenter (1965). The HCO_3^- efflux was measured titrimetrically according to Berger (see Karlgren

TABLE 1 The 4 combinations of respiratory gas regimes in the ambient water used in continuous bioassays lasting for 52 days. Daily determinations of O_2 in per cent air saturation, PCO_2 mmHg and pH are given as means and range.

Test group									IV		
I			II			III					
Moderate PCO_2						High PCO_2					
PO_2 next to air saturation			PO_2 reduced			PO_2 next to air saturation			PO_2 reduced		
O_2 of air sat.	PCO_2 mmHg	pH	O_2 of air sat.	PCO_2 mmHg	pH	O_2 of air sat.	PCO_2 mmHg	pH	O_2 of air sat.	PCO_2 mmHg	pH
Mean 89	8	7.6	49	8	7.6	87	23	7.1	49	23	7.1
Range 85-91	8.0-8.2	7.5-7.6	44-52	8.0-8.2	7.5-7.6	84-90	19.8-26.7	7.0-7.2	47-52	20.3-26.1	7.0

1964) and PCO_2 calculated on the basis of alkalinity, pH and temperature using the constants of Höglund (1961).

Blood sampling and autored graphs. Just before starting the exposures (day 0 in the bioassay) fish was given one standard dose of $1 \mu Ci$ ^{59}Fe by i.p. injection (0.1 μg Fe as ferric citrate complexed with sodium citrate made isotonic with NaCl, Amersham England 1971 code IES-1P). Blood was sampled after the exposures of 3, 6, 10, 15, 20, 30, 41 and 52 days for autoradiography on blood smears.

Electrophoresis. At the intervals mentioned above blood was also taken from two fish from each group for electrophoretic separation of "mature hemoglobins" (cf Koch 1972). The erythrocytes were washed in 0.9% NaCl, hemolyzed in distilled water and electrophoresis was carried out on each fish using Tris/HCl buffer, pH 8.3 as described by Koch *et al.* (1964). The prepared electrophoretograms were pressed against Kodak Red Seal X-ray films and exposed for 15 days at 0-4°C and then developed in Kodak D19B.

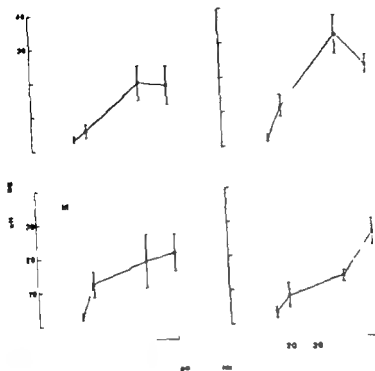
Light microscope and the classification of RBC as to incorporation of radioactive iron and the degree of maturation. The preparations of blood smears were examined under light microscope at the surface of 720 times. The incorporation of radioactive iron and the classification of red cells as "mature" and "immature" RBC of Classes 1-4 according to Härdig (1978) dealing with the same primary observation were made. Accordingly the remaining morphological types of RBC belonging to Classes 5-6 (Härdig 1978) are designated "mature" RBC. The percentages of immature RBC of all cells examined have been calculated as well as the proportions of labelled cells. The groups scored as immature and mature erythrocytes. The calculations are based on approximately 100 RBC examined from each blood smear. Blood smears were taken from seven fish at the exposure times chosen. Test groups I and III and from 5 fish at the end of the test in Test groups II and IV. Two blood smear preparations were obtained from each fish.

Statistical treatment. For the sake of simplicity the regressions calculated for ascending parts of the curves (Day 15-41) in Fig. 1, 3 (Table II) were considered to be linear. In Fig. 2 comparisons were made within experimental groups. Otherwise comparisons between test groups and control group have been accomplished regarding pairs of experimental series. All significance levels have been determined with Student's t-test.

Results

A. The categories of RBC and hematological parameters studied

In young salmon immature RBC delivered to the circulation exhibit continuing morphometric changes. In the present study the RBC were categorised as belonging to two age groups, i.e., an earlier one without and a later one with iron uptake since the start of the assays and the simultaneous administration of ^{59}Fe . Thus during the present exposures in



1. Changes in the proportion of labelled red blood cells (mean values and S.D. for $n=5-7$) in per cent of RBC existing during the course of exposures to four respiratory media (see Table I). Each test fish gives one sample dose of ^{59}Fe at zero time. Constants for regression lines calculated on the same results obtained from Day 13 until Day 41 are given in Table II.

low respiratory gas media, the RBC—if incorporating the radionuclide—after entering blood stream, pass through the unlabelled and then through the labelled maturation compartment into the labelled mature compartment.

The following four categories of RBC have been dealt with 1) labelled RBC (Fig. 1); 2) immature RBC (Fig. 2); 3) immature labelled RBC (Fig. 3); 4) mature labelled RBC (Fig. 4). The levels of the fractions of these categories have been followed after the administration of ^{59}Fe in order to obtain data as to the sizes and transit times of the two maturation compartments discerned. The flow out of the maturation compartment was measured by the changes of the levels of mature labelled RBC with time.

The increase of the RBC pool in the maturation compartments is caused either by a new formation, inflow from RBC depots and/or by elongated transit time, i.e. a more or less temporarily delayed maturation.

1. Erythropoietic events in each respiratory environment tested

Comments on the present control condition of Test group I In the control fish (Test group I in Table I) kept in water with high oxygen (corresponding to 90% air saturation)

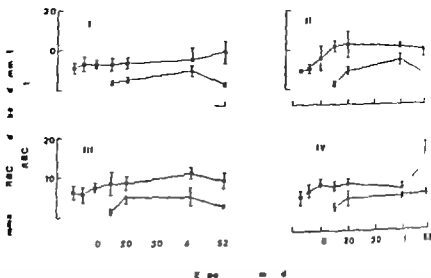


Fig. 2. Changes in the percentages (mean values and S.D. for $n=5-7$) of immature RBC (■) of all fish examined at 7 occasions and changes in the percentages of labelled immature RBC (▲) studied at variously long exposures to each of 4 combinations of oxygen content and carbon dioxide tension in ambient water (Table I). At the start of the tests the fish were given single doses of ^{59}Fe .

The increase in the percentage of immature RBC as compared with that for Day 3 was determined within each group. Significance levels were $0.01 < p < 0.05$, $0.001 < p < 0.01$ and $p < 0.001$.

and low carbon dioxide tension (PCO_2 8 mmHg), after a delay until Day 15 the percentage of labelled erythrocytes increased from 3 per cent at Day 15 to 19 per cent at Day 41; then it levelled off (Fig. 1 Table II).

The proportion of immature RBC, (*i.e.* the sum of labelled and unlabelled cells as a per cent of all cells examined) did not change significantly during the test period (Fig. 2). The pool size of labelled immature RBC on the other hand, increased during the part of the test lasting from Day 15 to Day 41. After that it declined until the end of the test at Day 52 (Fig. 2). This final decrease most likely was due to diminishing contents of ^{59}Fe in the body iron stores.

With the limitations discussed on p. 247 the data in Fig. 2 may permit by extrapolation an approximate estimate of transit time for labelled immature RBC. In the Test group I it amounts to about 20 days. The constants of the regression lines presented in Table II can be used to estimate erythropoietic activity measured as the increase in percentage of mature labelled RBC per day. This will give a numerical value of 0.51 valid for the control Group I.

2. Activation by lowered PO_2 in Test group II In the fish kept in water with a reduced oxygen content, the fraction of labelled RBC increased at a significantly higher rate than in the controls (Fig. 1 Table II).

The proportion of immature RBC increased rapidly from Day 3 to Day 20 and then it levelled off. Values significantly higher than in the control group were attained within the period from Day 15 to Day 41 (upper curve in Fig. 2).

At the examinations made after 20 and 41 days the level of labelled immature RBC was also significantly higher than in the control group and a decrease from Day 41 to Day 52.

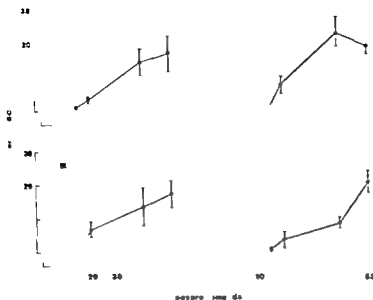


Fig. 3. Changes in the proportions (mean values and S.D. for $n=5-7$) of labelled mature RBC in young salmon after a single dose of ^{51}Cr at the start of 4 assays with different combinations of oxygen and carbon dioxide tensions in the respiratory water as shown in Table I. Constants for regression lines fitted on the observations from Day 15 until Day 41 are given in Table II.

is also seen here, presumably for reasons discussed above (p. 244). No exact determination of transit time could be done because the increasing proportion of immature RBC. As the total amount of immature cells was approximately the same Day 41 and 52, the slope of the line for labelled immature RBC for this part of the assay has been used to derive the transit time for labelled immature RBC. Then also in this case it will amount about 20 days (*cf.* Test group I). If this holds good the observed increase in immature RBC must be the result of an enhanced output of RBC, which will also explain the observed high level of mature labelled RBC, nearly reaching 20 per cent at Day 41 (Fig. 3).

As compared to the controls the erythropoietic activity was significantly enhanced in the test group II giving an increase in the percentage of mature labelled RBC per day of 0.80 (Fig. 3 Table II). Thus at pH/PCO₂ 7.6/8 pH units/mmHg an oxygen depletion from 90 to 0 per cent air saturation stimulated the erythropoietic activity in the present yearlings of salmon *salmo L.*, evidently by proliferating RBC stem cells as well as enhancing RBC maturation. This was manifested within a period of 20 days since the fish were submitted to the new environment.

3. *Effects of high PCO₂ in Test group III* In the assay with carbon dioxide supersaturated water there was a significant increase in the labelled cells during the whole test period (Fig. 1, Table II). The regression lines for the changes in the proportion of labelled RBC with time are not significantly separated from each other in Test groups I and III (see Table II). Thus no effects of the high PCO₂ was obtained in this parameter.

TABLE II Statistics for the correlation between assay time in days (from Day 15 until Day 41) and percentages of A) labelled RBC (Fig. 1) and B) labelled mature RBC (Fig. 3).

	x days	y per cent	Test group	n	b ^a	r ^b
A	Assay time	Labelled cells	I	17	0.63	0.94
			II	14	1.07	0.95
			III	19	0.55	0.74**
			IV	14	0.35	0.85
B	Assay time	Labelled mature cells	I	16	0.51	0.95
			II	12	0.80	0.95**
			III	16	0.44	0.81
			IV	12	0.28	0.80*

The regression coefficients (b) for Test groups II, III and IV have been compared with the test in control in Group I using t test.

^a Correlation coefficient r

The significance levels were $0.01 < p < 0.05$ $0.001 < p < 0.01$ and $p < 0.001$

The proportion of immature RBC increased with time as shown in Fig. 2 (II, upper curve) and the value for Day 41 was significantly higher than in the control group ($0.001 < p < 0.01$)

The proportion of labelled immature RBC changed as shown in Fig. 2 (III, lower curve) but no significant differences occurred when compared to the control group. Whether the transit time for immature RBC through the labelled maturation compartment was elongated compared to the control group cannot be exactly estimated from the present data. Extrapolation in Fig. 2 (III) however it may be approximated in Test group III to about 5 days.

The erythropoietic activity measured as the increase in the percentage of mature RBC per day amounted to 0.44 which is not statistically different from the value found in Test group I (Table II)

Neither were the increasing levels of immature RBC as compared to the control group (Fig. 2) accompanied by a significantly changed output of mature RBC (Fig. 3 Table II). In this group the high PCO probably acted on the proliferation of the stem cells in order to increase the rate of erythropoiesis. Probably by elongating the RBC maturation time the net output of mature cells is not higher than in the control group. Instead accumulation of unlabelled immature RBC may occur.

4. Combined effects of lowered PO and high PCO in Test group IV In Test group IV there was a significant increase in labelled RBC over the whole test period (Fig. 1 Table II). In this case the regression for the increase in the proportion of labelled RBC from Day 15 to Day 41 was significantly lower than in the control group (Table II)

The level of immature RBC increased as shown in Fig. 2 (IV) and a significantly higher value was found for this parameter at Day 10, 15, 20 and 52 when compared to Day 1. From Day 41 to 52 a significant increase was also obtained $0.01 < p < 0.05$. Compared to Test group IV with the control group (I) the parameter dealt with in the upper curve of Fig. 2 differs significantly between groups for Day 52 only ($0.01 < p < 0.05$)



4. Electropherograms (above) and autoradiograms (below) of hemolysates from control fish living in Test group I in Table I. Time after the injection of ^{55}Fe was 15, 22, and 41 days in I and C, respectively. Essentially the same is was found at regarding Test groups II, III and IV on all days of observation.

Contrary to the results in Test groups I-III no decrease in the level of labelled immature C was found in Test group IV from Day 41 to Day 52. The turnover of iron thus was as in the latter group and the transit time for immature RBC prolonged.

As presumed in Fig. 3 (IV) and Table II also the output of labelled mature RBC was totally lowered in this group until Day 41. Then a rapid increase in the percentage of labelled mature RBC started. The estimate for erythropoietic activity from Day 15 to 41 gives a numerical value of about 0.28 per cent increase in labelled mature RBC per day (Table II). This evidently also points to an impaired erythropoiesis.

Incorporation of ^{55}Fe and ^{59}Fe into hemoglobin pattern

The autoradiograms for the electropherograms in Fig. 4 shows that ^{55}Fe was present in all hemolysate fractions from Day 15 until Day 52, i.e. from the time when radioactive iron became detectable within the red cells.

Discussion

Shortcomings in methodological respects

The growth rates of test fish was not examined and the changes in blood volume could not be controlled. There was great variation in fish body weights but no correlation was found between the amount of immature RBC and fish weight in any group at any day of sampling. The estimations made here of pool sizes and transit times regarding the two maturation compartments can only be done provided that the new formation of RBC and the maturation rate are constant during one experiment which apparently is not true.

The experiments were designed to be cohort labelling (Berlin 1964). The percentages of labelled mature RBC were thus expected to reach plateau and then to decline. As seen in Figs. 1 and 3 this did not occur. Inadequate amounts of administered ^{55}Fe , too short

duration of the tests as well as increased erythropoietic rate and/or RBC volume may have contributed to this discrepancy.

B Starting point for the incorporation of radioactive iron

Härdig (1978) found that the iron uptake of RBC was completed before reaching the mature stage. In the present study no incorporation of the injected iron could be detected in RBC considered to be mature at the time for the administration of radioactive iron. This indicates that mature cells did not start new hemoglobin synthesis in response to the present hypoxia or other environmental conditions used in the assays.

From fish exposed for 3 and 6 days in either test group none, and after 10 days even still very few labelled cells appeared in the autoradiographs. Thus labelled cells were falling during early parts of the tests as shown by the presentations in Table II and Figs 1-3. The lag phases in the appearance of labelled RBC probably reflect the time needed for the incorporation of administered radionuclide in the iron pools and to reach sufficient grain counts above the labelling level chosen (cf p. 242).

C Morphology and life span of fish RBC

The oldest morphological type, or senile RBC discerned in the carp by Topf (1955) could not be recognized here, but all other stages in the development series of RBC described by him were identified (Härdig 1978). The absence of senile RBC might be explained by the postulate that the RBC life span exceeds the age of the fish material and also the exposure time used in the present study. Alland and Bruce (1962) report that the mean life span of turtle RBC probably is 600-800 days as compared to 700-1400 days for turtles (*Anura*).

D Evaluation of erythrokinetic responses induced

It appears from the present results in Fig. 2, Test groups II, III and IV in comparison with the Control group I that some effective mechanism(s) exist which act upon the erythropoiesis in response to the respiratory gas parameters PO_2 , PCO_2 and/or pH. This indicates that the proliferation of RBC stem cells were stimulated.

The erythropoiesis was activated within a few weeks by the present reduction of oxygen content in the respiratory water which caused an increase in the RBC maturation pool and enhanced the output of mature RBC (Fig. 2 and 3, Table II). This verifies earlier findings, i.e. by Zanjanli *et al.* (1969) in another species of fish (*Trichogaster trichopterus*).

The carbon dioxide oversaturation in Test group III did not change the output of mature RBC (Fig. 3, Table II). Rather a retardation of RBC maturation may occur. This should be considered in connection to the findings by Börjeson and Höglund (1976), that sustained CO_2 oversaturation almost equal to that in the present test conditions of Group III (cf Höglund and Härdig 1969, Höglund and Börjeson 1971, Höglund and Pernow 1971) causes a pronounced Root effect which in young *Salmo salar* is transient and parallel to the acidosis initially caused by the diffusion of carbon dioxide from the environment into the blood. By compensatory acid-base regulation it fades out within 30 days (Börjeson and Höglund 1975, 1976, Börjeson 1976, 1977).

environment in Test group IV acted negatively upon the fish. The transit time for one RBC was elongated and the output of mature RBC lowered. This is presumably disturbed metabolism on account of starvation and initial strain. It is known from fish, that an inadequate metabolic environment elongates the maturation time of erythropoiesis (Harris and Keffenmeyer 1972). Zanjani *et al* (1969) also report that in the blue gourami (*Gambusia melanocephala*) the erythropoiesis is inhibited by starvation. Now in the case of young salmon in Test group IV the specimens did not feed well until the fourth or fifth week of the assay.

Application of the present autoradiography on multihemoglobins

The electrophoretic pattern of so called multihemoglobins in salmon (Koch *et al* 1964) is known to depend on fish size, age, race and breed or physiological condition (Hashimoto and Ueda 1962, Wilkins 1968, Wilkins and Iles 1968, Koch 1972). That is why electrophoresis on blood haemolysates were performed to possibly find correlations with the quality of water conditions chosen. However the present respiratory stress conditions did affect the "multihemoglobin" pattern in the young *Salmo salar* tested. Thus, our results fully support the view expressed by Giles and Varstone (1976) who were not able to induce changes in the pattern of multiple hemoglobins from coho salmon (*Oncorhynchus kisutch*) fry and pre-smolts by varying PO_2 .

The intention was also to identify the biochemical background to the electrophoretic pattern, which though using various electrophoretic and gel filtration techniques has not yet been unsuccessful. As ^{59}Fe incorporated in RBC presumably is bound to hemoglobin, it cannot be decided to what extent the recorded radioactivity is due to hemoglobin or other ferroproteins such as cytochromes, catalases or peroxidases. Whether or not the salmon "multihemoglobins" are correlated with fish development and maturation could not be determined, as radioactivity appeared in all fractions separated from 15 and onwards.

Then, to summarize, the diagnostic value of changed "multihemoglobin" pattern in fish is questioned. The physiological emphasis and correlation to erythropoiesis of these "toxic" fractions are still obscure.

Hematology in testing health condition in fish

Sprague (1976) criticizing sublethal tests of pollutants on fish referring to McKim *et al* (1970) points out that the effects of copper on five blood parameters in *Salvelinus fontinalis* are either transient during 1-year exposures or less sensitive than effects on reproduction, growth and survival. This and other reviewed facts led Sprague (1976) to the general conclusion that laboratory blood physiological tests have yielded disappointingly few water quality criteria concerning the large number of investigations.

The main result of the present tests supports this view. A general test (like the sedimentation rate reaction in human medical diagnostics) to apply blood parameters in examining the state of physiological efficiency, injury or well being of fish is still missing.

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Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed

By

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Abstract

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Histamine infused intra arterially into artificially perfused, maximally dilated rat hindquarter vessels increased fluid filtration and CFC but had essentially no effect on the diffusion capacity to small solutes. Isoprenaline largely prevented the increase in fluid filtration and CFC if infused prior to the start of the histamine infusion and if infused after the start of the histamine infusion, promptly reduced fluid filtration and CFC to near control levels. Additionally it was noted that severe cooling of the perfused hindquarter largely prevented the marked increase in fluid filtration and CFC by histamine. The antagonism of isoprenaline induced increases in macromolecular permeability represents a direct action of isoprenaline on the microvascular membrane which effectively counteracts that of histamine. The data also suggest that the large pores created by histamine are different from the large pore through which macromolecules normally transverse the microvascular membrane and that catecholamines may exert a regulatory function in the control of microvascular permeability to macromolecules in pathophysiological states associated with massive histamine release.

Although the concept that the sympathetic nervous system and the circulating hormones adrenaline and noradrenaline may affect microvascular permeability to both water and macromolecules was proposed decades ago there followed little convincing evidence to support this hypothesis. These early studies (Engel 1941 Asher and Jost 1914) purported that the sympathetics affect microvascular permeability were subjected to severe criticism (Chambers and Zweifach 1947 Danielli and Stock 1946) as the experimental approach to the problem was indirect and the investigators often failed to consider changes in hemodynamics and Starling capillary forces in the interpretation of their results. Attempts to substantiate these conclusions in better controlled studies were uniformly unsuccessful (Cheng 1949) that is, no measurable effect of the sympathetics on capillary permeability could be demonstrated. Subsequently it became universally accepted that normal microvascular permeability including that to macromolecules, was not under the influence of the sympathetic nervous system or circulating catecholamines (Folkow 1955).

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...over years some evidence has accumulated suggesting that in certain vascular beds, under certain conditions, this generalization may not be true. In canine adipose tissue, simple, sympathetic nerve stimulation increases microvascular membrane permeability (Rowell 1967, Rowell, Intaglietta and Chisholm 1974, Linde and Calver 1974, Chisholm and Rowell 1974). The possibility that sympathetic stimulation in this bed causes the liberation of histamine-like substances has not been excluded. Hence, increased microvascular permeability in adipose tissue in response to nerve stimulation represents an indirect rather than a direct effect of the sympathetics. There is no reason that sympathetic stimulation increases microvascular permeability in other canine vascular beds.

It has, however, recently been demonstrated that the massive protein efflux caused by intra-arterial infusion of histamine into the canine forelimb can be largely prevented by systemic hypotension (Marchinlak *et al.* 1977) or alternatively by a simultaneous intravital infusion of noradrenaline or isopropyl noradrenaline (isoprenaline) (Marchinlak *et al.* 1977). This antagonism of histamine protein efflux by prior systemic hypotension or by catecholamine infusions could not be attributable simply to decreases in blood flow, since it also occurred during constant flow conditions. Evidently the sympathetic neuro-modes could reduce the microvascular transfer of macromolecules under conditions when the permeability to macromolecules in canine forelimb skin and skeletal muscle has been greatly increased by histamine. However a reduction in perfused surface area could contribute to this effect and the extent of such a contribution remains to be determined. In view of the possible importance of catecholamines as physiological antagonists of histamine and bradykinin (Maciejko *et al.* 1977) in the microcirculation, it was decided to study in more detail the histamine-catecholamine microvascular permeability interaction under conditions when both diffusion and convection are simultaneously followed, utilizing an artificially perfused isolated rat hindquarter preparation as the test organ. One major aim of this study was to determine if the antagonistic action of catecholamines on the histamine-induced increase in protein efflux reflected a direct action on the microvascular membrane alone or if also decreases in perfused surface area contributed importantly to this effect. It was further considered important to determine if this was a species-dependent effect as earlier studies were all performed on dogs.

Methods

Hypotensive and arterial arrangements. Experiments were performed on the isolated hindquarters from 3 male Wistar albino rats, weighing between 270 and 380 g. The hindquarters were arranged for the 'isopermeatic' method (cf. Pappenheimer and Zouh Rivers 1948, Eliaeson *et al.* 1974) and for 'colormetric' or 'tail' modification of the single injection indicator diffusion method (Rippe and Stage 1976, 1977).

The isopermeatic rat hindquarter preparation has been described elsewhere (Folkow *et al.* 1974). Briefly, the tail artery was cannulated during ether anaesthesia for continuous intra-arterial pressure (P_a) recordings after which the animal was further anaesthetized with Nembutal (3 mg/100 g, and etherized). The abdominal aorta and the inferior vena cava were freed from the level of the renal vessels to the abdominal aorta. Then the hindquarters were isolated just proximal to these latter vessels by mass ligatures and the blood clamped and bone marrow was plugged with cotton wool soaked in silicone. Tail and paw were excluded by tight ligatures, so that the preparation consisted mainly of skeletal muscle (70-75%). After reperfusion the abdominal aorta was cannulated and connected to an artificial perfusion

system, driven by a Harvard perfusion pump. The caval vein was connected to a venous outflow cannula, while one of the renal veins was used for continuous venous pressure recordings (P_V). The completely isolated hindquarter preparation was then placed on a balance plate, connected to a strain gauge for continuous weight recordings. Finally the venous outflow cannula was coupled in series with a perfusate and a densitometer system for simultaneous recording of Cardio-Green albumin and Cr-EDTA. An arterial inflow cannula was side-branched to a step dispenser syringe for injection of the measured indicators, for measurements of capillary diffusion events (cf. Rippe and Stage 1978). The venous outflow cannula, i.e. the one distal to the densitometer system, could be adjusted to an arbitrary level to set P_V . Flow and P_A could be controlled by adjusting the setting of the perfusion pump. Its weight, P_A and P_V were continuously monitored on a Grass polygraph, while pH and the oxygenation were recorded on a potentiometer writer.

Four per cent dextran (Macrodex® MIV about 70 000 supplied by AB Pharmacia, Sweden) in Tyrode was used as perfusate to which was added either horse serum (Normal Serum, SBL, Sweden) or bovine albumin (Fraction V Powder Sigma Chemical Company USA), 6 g/l. The perfusate was pre-equilibrated with a mixture of 97% O_2 and 3% CO_2 and kept at 38°C. Perfusion flow was kept constant at 100 ml/min. 100 g of tissue throughout the experiment. Maximal vasodilatation was maintained during the whole experiment by means of slow infusion of papaverine.

Capillary filtration and diffusion were continuously followed in these experiments. Capillary hydraulic permeability was determined as capillary filtration coefficient (CFC) (cf. Eliasson *et al.* 1974). Large molecule permeability affecting the colloid osmotic pressure difference across the capillary walls, could be followed by the consequent changes in capillary fluid equilibrium, which caused net weight changes when the hydrostatic capillary pressure was kept constant. Small molecular permeability was assessed by measuring P_{Cr} for Cr-EDTA by a colorimetric modification of the single injection indicator diffusion method (Rippe and Stage 1978).

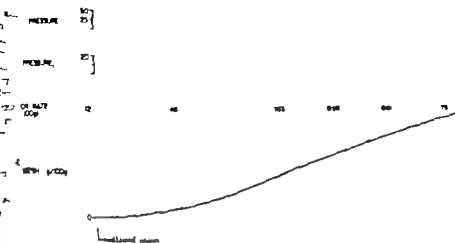
Experimental procedure

1 Action of Isoprenaline The histamine stimulated hindquarter vascular bed. Initially CFC and P_{Cr} for Cr-EDTA were measured 2-3 times while the hindquarter vascular bed was kept at complete isoprenaline for at least 15 min. Histamine, 30-60 μ g/ml of perfusate, was then infused into the preparation with changing flow. P_A in the already maximally dilated vascular bed. The concentrations of histamine used in this study were kept well above 15-20 μ g/ml, which was tested out to be the concentration required to obtain definitely maximal increases in microvascular permeability. This relatively low responsiveness of the rat to histamine, compared with some other species, is in agreement with earlier studies (e.g. Fox and W. yland 1976). All 11 animals responded to histamine but only 6 with pronounced increases in CFC and with marked net shifts of fluid caused considerable weight increase. To these six preparations were maximal doses of isoprenaline 30-70 μ g/ml of perfusate ($1.4-1.9 \cdot 10^{-4}$ M) were infused about 10 min before the start of the histamine infusion, so that the two drugs were now supplied simultaneously to the preparation. CFC, capillary fluid balance and P_{Cr} for Cr-EDTA were continuously followed during these experiments (cf. Results).

2 Action of histamine on the isoprenaline pretreated hindquarter vascular bed. Another four animals, which in other respects were treated in exactly the same way as the above mentioned ones, were infused with isoprenaline, going on for some 10 min before the infusion of histamine was started in parallel. Capillary permeability characteristics were followed as described above.

3 Approximate dose levels of isoprenaline required to suppress histamine induced permeability changes. In order to obtain a rough estimation of the dose levels of isoprenaline required to partially or completely abolish the histamine induced changes in microvascular permeability the following procedures were employed. To four hindquarters perfused with dextran and "aged" horse serum (see Results) supramaximal histamine infusions were first given for 8 min and the permeability changes were followed with CFC measurements. Subsequently an infusion of isoprenaline was given simultaneously with the histamine infusion in increasing concentrations, from 0.5 to 1 000 μ g/ml perfusate ($1.4 \cdot 10^{-6}$ to $7 \cdot 10^{-4}$ M) in three steps during a total period of 15-20 min. At every dose level CFC was measured and could be compared to the values obtained during the pure histamine infusion period. All measurements were performed about 25 min after the start of the histamine infusion, because in some experiments in which only histamine was infused a spontaneous decrease of CFC could be seen at this time (unpublished results).

4 Action of histamine on the cooled hindquarter vascular bed. In 6 animals CFC was first measured during control at normal tissue temperature (36-37°C). The tissue temperature was then reduced to between 10 and 15°C by means of cooling the perfusate to around 5°C. After CFC measurements at 10-15°C the cooled



1. The effect of incremental doses of histamine (30 μ g/ml, max. 100 g) on the capillary fluid balance, expressed by changes in tissue weight in the non-vascularized hindquarter vascular bed. The tissue weight is taken from preparation. Here CFC measurements were made after the time period as in the Figure. The filtration rate measured 4-8 min after the start of the histamine infusion is all in CFC values obtained 15 min later are approximately the same as those in the next, shown in Fig. 3 at the isoprenaline infusion started.

hindquarter preparation is kept at isogravimetry for some 10 min after each histamine, 30-60 μ g, ml infusion was infused as described above. The histamine infusion was continued for 20 min and changes CFC and capillary fluid balance were registered. For an additional 15 min after stopping the histamine infusion the preparation was kept at 36-37°C, after which it was returned to 37°C by means of increasing the perfusion temperature to 38°C. After control CFC measurements and a new period of isogravimetry, the tissue was again infused into the preparation in exactly the same dose as earlier and the effects on CFC and tissue weight were studied.

Results

Effect of isoprenaline on the histamine stimulated hindquarter vascular bed. In the 6 tested hindquarters (see above) histamine caused an increase in CFC from 0.0483 ± 0.0030 ml/min mmHg 100 g (mean S.E.) at control, to 0.1315 ± 0.0019 ml/min mmHg 100 g. Under the influence of histamine the preparations also started to gain weight, i.e. to deviate from state of complete isogravimetry and this usually occurred within 2 min from the start of the infusion. In the 6 preparations mean filtration rate amounted to 0.86 ± 0.11 ml/min 100 g 5-8 min after the start of the infusion (see Fig. 1 and 2).

During the histamine-isoprenaline infusion CFC rapidly returned to near control 0.0510 ± 0.0013 ml/min mmHg 100 g ($p < 0.001$) and the filtration rate decreased significantly to 0.18 ± 0.03 ml/min 100 g within 5-10 min ($p < 0.01$) (see Fig. 2 and 3).

After stopping the isoprenaline-histamine infusion the preparations seemed to be absolutely refractory to histamine for the following 30-60 min and relatively refractory to isoprenaline for another 1 or 2 h. PS for Cr EDTA changed only slightly from control values (3-7%) during the histamine infusion period or when both histamine and isoprenaline infusions were performed. This is in agreement with recently published results from this laboratory. The PS values measured during the histamine infusion period seem to have

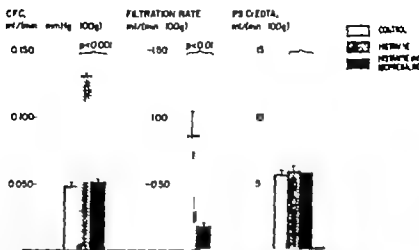


Fig. 2. CFC, filtration rate and PS for Cr EDTA (mean \pm S.E.) in the maximally isolated hindpaw preparation at control, some 3-8 min after the start of the histamine infusion and around 18 min after start of the combined histamine-isoprenaline infusion.

mainly reflected the diffusional exchange of Cr EDTA and only to a very limited extent conventional exchange. The reason is that histamine increases the albumin concentration in the filtrate to near plasma levels (Appelgren, Jacobson and Kjellmer 1966). Thus the non-permeant tracer (C-G albumin) is filtered through the exchange vessels at about the same rate as water. Consequently the increase in relative concentration of the non-permeant tracer in the venous affluent normally seen during filtration is very slight and the measured extraction fraction now greatly underestimates the bulk flow contribution in the inter-

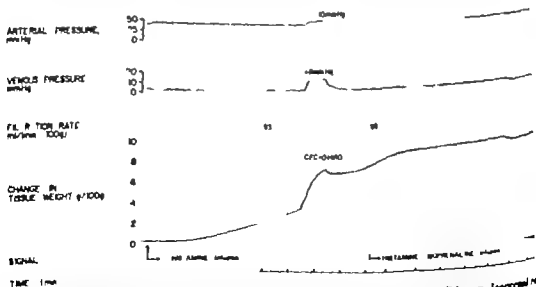


Fig. 3. The effect of isoprenaline on histamine induced changes in capillary flow and filtration rate. Isoprenaline changed the rate of filtration during histamine infusion from 0.91 ml/min 100 g of tissue to 0.11 ml/min 100 g of tissue in 2.5 min. In this expt. control CFC was 0.413 ml/min mmHg 100 g. Here the CFC value during histamine is shown in the figure. The CFC value measured 10 min after the start of the histamine-isoprenaline infusion was 0.0436 ml/min mmHg 100 g. Flow P_A and P_V were held constant throughout the recording with exception made for the period of CFC measurement.

ort. In the present histamine expts. this was calculated to at maximum 7-8% of total ort.

5 animals, which responded with only "moderate" changes in CFC (50-150% increase) comparably low filtration rates (0.25-0.90 ml/min 100 g) to histamine, and which excluded from the further investigation, since it was deemed desirable to have initially no histamine effects, were all perfused with fresh horse serum in dextran-Tyrode solution. Full histamine responses could not be obtained unless bovine albumin or aged serum (stored for 3 weeks at 4°C) was used instead of fresh horse serum in the etc.

Action of isoprenaline on the isoprenaline pretreated hindquarter vascular bed. It was a new finding that pretreatment with isoprenaline rendered the hindquarter vascular bed insensitive to histamine, and this effect lasted for at least 60 min after stopping isoprenaline infusion. Thus, no significant changes in fluid filtration CFC or in PS for Cr-EDTA are observed during the isoproterenol infusion or during the isoproterenol-infusion period.

Approximate dose levels of isoprenaline required to suppress histamine induced permeability. Complete inhibition of the histamine-induced changes in microvascular permeability as in 4 expts. obtained at isoprenaline concentrations of 40-70 ng/ml perfusate (mean 52 ng/ml), but partial suppression of the histamine effects was seen already at around 10 ng/ml perfusate ($\sim 3 \cdot 5 \cdot 10^{-9}$ M) of isoprenaline. It should then be stressed that this is the case despite the use of supramaximal histamine concentrations.

Action of histamine on the cooled hindquarter vascular bed. Control value of CFC before cooling was 0.0425 ± 0.0016 ml/min mmHg 100 g, which is somewhat lower than value given above. The difference is probably due to differences in perfusate, because in present group only aged horse serum was used as colloid in the dextran-Tyrode solution and in the histamine-isoprenaline groups half of the expts. were performed with bovine albumin in the perfusate. With the latter perfusate control CFC were always higher than in the former. At 10-15°C CFC decreased to 0.0281 ± 0.0010 ml/min mmHg 100 g ($p < 0.001$). Peripheral vascular resistance increased from 2.98 ± 0.06 mmHg ml min 100 g to 4.51 ± 0.11 mmHg ml min 100 g. The reaction of the cooled microvascular bed to histamine was delayed and markedly depressed. During the first 5-7 min of histamine infusion the cooled preparation was almost completely isogravimetric as shown in Fig 4 upper part but it then started to filtrate at a low rate, CFC increased some 50-60% to 0.043 ± 0.0164 ml/min mmHg 100 g ($p < 0.06$). After stopping the histamine infusion and after rewarming CFC returned to control value. Histamine stimulation now produced a almost threefold increase in CFC and also marked filtration. The changes are summarized in Fig 5.

Discussion

Histamine markedly increased CFC and caused net fluid filtration in the initially isogravimetric rat hindquarter preparation. As the vasculature was already maximally dilated, indicated by the failure of histamine to further change arterial and venous pressure or the PS for Cr-EDTA, the changes in CFC must be exclusively attributable to change in

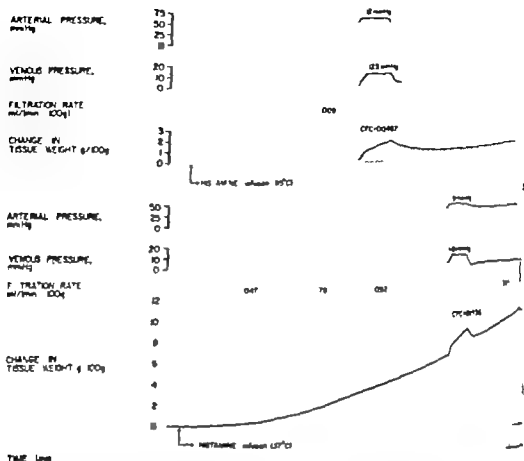
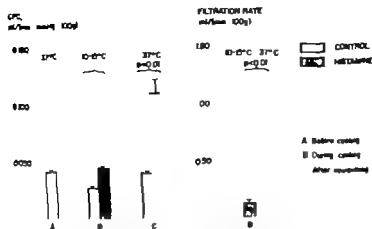


Fig. 4 CFC and capillary fluid equilibrium after histamine during cooling (upper part) and after rewarming (lower part) 30–40 min later in the same experiment. The arterial pressure in this experiment was around 37 mmHg during cooling and around 37 mmHg after rewarming while flow was kept constant (1–2 ml/min 100g) as well as the venous pressure (13 mmHg).

microvascular permeability rather than to any change in perfused capillary surface area. For essentially the same reasons the histamine-induced increase in net fluid filtration could be attributed to changes in microvascular hydrostatic pressure and surface area, but not reflect a decrease in the transmural colloid osmotic pressure difference, caused by an increased permeability to macromolecules. However, despite these large changes in microvascular hydraulic conductivity and permeability to macromolecules, permeability to small molecules was not significantly altered from control during the histamine infusion. This is inferred from the PS values for Cr EDTA which failed to change significantly in response to histamine as more extensively dealt with in another study already published as a preliminary communication (Rippe, Kamly and Folkow 1978). This discrepancy can best be explained by a preferential action of histamine on venular gap formation (large pores), resulting in an increase in their number and possibly their radius. The large pore to small pore ratio seems to be approximately 1 to 30 000 (Grotte 1956) up to 1 million (Winne 1965, Renkin 1970) of the total population of pores. By virtue of their big radius, 25 nm (Grotte 1956) up to ~160 nm (Renkin, Carter and Joyner 1974), the large pores are of particular importance for the movement of macromolecules from blood to interstitium and also for hydraulic



The effect of cooling and subsequent rewarming on CFC during control and during supraphysiological dose infusion (means \pm S.E.) (left part). The maximal filtration rates during histamine infusion, during cooling and during subsequent rewarming are also shown (right part).

activity as this increases with the fourth power of the radius. However, as a result of small total cross sectional area relative to the small pore system the large pores exert less influence on the diffusion transfer of small molecules, which changes only with the third power of the radius. For such reasons, small molecular permeability would be only slightly affected even if the number of and radius of large pores is markedly increased. For example, if their number is increased 30 times CFC would theoretically increase 3 to 4 times and the microvascular filtrate concentration of albumin about ten times (cf. Appelgren, Robinson and Kjellner 1966), while the increase in effective pore area for small molecular fraction would be less than 10% calculated from Grotte's data (Grotte 1956).

The marked increase in CFC caused by histamine could be largely prevented by the simultaneous infusion of the catecholamine isoprenaline. Since all hemodynamic factors as well as the diffusion capacity for small molecules were essentially unchanged relative to control during the combined histamine-isoprenaline infusion, a decrease in microvascular surface area cannot have contributed to the failure of histamine to increase CFC under these conditions. Instead, it is more likely that isoprenaline prevents the histamine-induced increase in microvascular permeability to macromolecules by a direct action on the microvascular membrane which counteracts that of histamine. This counteracting of histamine's macromolecular efflux by isoprenaline is apparently a physiological antagonism and not a matter of histamine receptor blockade. It has been reported that histamine increases the number of large pores in the immediate postcapillary venules by causing adjacent endothelial cells to "round up" thereby creating gaps presumably as a result of contraction of actomyosin-like anchoring filaments in the endothelial cells (cf. Haddy Scott and Grega 1976). As the antagonism by isoprenaline does not involve histamine receptors, it is logical to assume that isoprenaline via beta-adrenergic receptors causes relaxation of these filaments counteracting the contracting action by histamine. Svensjo, Persson and Arfors (1976) noted in a preliminary report that the marked increase in the number of venular leaks of FITC-dextran

(MW 150 000), caused by topically applied bradykinin, could be greatly reduced by simultaneous topical application of the beta 2 adrenergic receptor agonist Terbutaline. The leaks are only found in the venules and represent gap formations between adjacent endothelial cells. As bradykinin acts similarly to histamine with regard to the site and nature of macromolecular efflux, it is reasonable to accept a similar hypothesis for the acceptance of histamine macromolecular efflux by catecholamines.

The great increase in CFC while PS for Cr EDTA is essentially unchanged upon later administration fails to support recent suggestions (Renkin, Carter and Joyner 1974, Crayth, Joyner and Renkin 1974) that histamine should primarily enhance vesicular transport rather than increase the number of large pores, because in such a case changes in CFC could hardly be expected (cf Renkin 1977). Further Rippe, Kamiya and Follow (1978) demonstrated that marked cooling of the vasculature of the rat hindquarters via its perfusion failed to alter the clearance of macromolecules subsequent to increases in venous pressure relative to the situation in a parallel perfused preparation kept at 37°C. If transendothelial protein transfer really occurred by means of micropinocytosis, which at least in some of the links must represent energy dependent processes, the above findings would be impossible to explain while they are in precise agreement with macromolecular transfer via large pore filtration. In addition as noted in this study severe cooling markedly reduced the extent of the increase in microvascular permeability to macromolecules and CFC by histamine an effect that was immediately reversible by rewarming the perfusate to 37°C. When considered together these data suggest that the microcontractile effect on venular endothelial cells by histamine is temperature sensitive and involves opening of intra-endothelial gaps other than the large pores responsible for the normal transfer of macromolecules. Furthermore, histamine can hardly exert these effects uniformly on the small pore system as this would invariably be reflected by changes in small molecular transfer by means of diffusion which was not the case.

The effects of severe cooling on antagonizing the histamine actions on the venular membrane could explain the beneficial effects of "ice packs" in the treatment of various inflammatory and burn responses. It has been assumed that the marked reductions in tissue swelling by severe cooling simply reflected decreases in blood flow into the injured tissue. Perhaps an equally important action of severe cooling results from an antagonism of the microvascular actions of histamine and other mediators of inflammation by cold diminishing the development of venular gaps which would greatly reduce protein and fluid loss.

Additionally the data in this study demonstrate that isoprenaline antagonizes the action of histamine on macromolecular efflux even if given after the onset of the histamine infusion, at which time the effects of histamine are already maximal and remain so as long as the histamine infusion continues. Shortly after initiating the isoprenaline infusion, CFC returns to nearly control levels. It was also observed that after exposure of the rat hindquarters to isoprenaline, the microvascular membrane remained relatively refractory to histamine for 30 to 90 min despite the fact that isoprenaline was not infused during this time. Relative refractoriness to topically applied bradykinin for 30 to 90 min was also observed in the hamster cheek pouch after exposure to isoprenaline (Grega, to be published).

It is intriguing to speculate on the possible physiological significance of these unique

of catecholamines on the microvascular membrane. While there is still no evidence that catecholamines are normally involved in the regulation of microvascular ability to macromolecules, this action of catecholamines to reduce permeability would paramount importance mainly in patho-physiological states associated with wide and massive histamine and bradykinin release. These often shock-like states are associated with profound hypotension which would function as a stimulus for an adrenergic-adrenal discharge. Additionally high concentrations of histamine and these elicit a direct release of catecholamines from the adrenal medulla. The anesthetic action of the catecholamines could prevent serious circulatory derangements from protein and fluid loss. In contrast, during local inflammatory responses, the actions of these endogenous agents would be unopposed. These data could explain, in part, several findings by perplexing observations. In contrast to local intrarterial infusions of histamine in the dog forelimb systemic infusions of histamine either intravenously (Grega *et al.* 1975) or into the left cuticular chamber (Marciniak *et al.* 1977) fail to promote edema formation and, instead, produce sustained net extravascular fluid reabsorption in the canine limbs. This route-dependent differential action of histamine cannot largely be explained by hemodynamic differences or differences in blood concentrations of histamine as a function of the route of administration (Marciniak *et al.* 1977 a), and, therefore, must represent a question of the histamine action on the microvascular membrane. Further also in the rat hindquarters perfused with horse serum, the ability of histamine to increase filtration and CFC varies directly with the age of the horse serum. The effect of infusion of histamine is reduced during perfusion of the rat hindquarters with fresh horse serum. In contrast, histamine exerts marked effects on microvascular permeability to plasma proteins and CFC when perfused with aged horse serum (that which has been stored for 2-3 weeks). It is tempting to speculate whether this discrepancy might be due to the possibility that aged horse serum contains liberated catecholamines, which may antagonize the actions of histamine on the microvascular membrane and that these factors might be slowly destroyed during the period of storage. Additionally other biogenic agents present in the serum might also function as physiological antagonists of histamine and contribute significantly to these characteristic actions of histamine.

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Aspects of temperature regulation in harp seal pups evaluated by *in vivo* experiments and computer simulations

By

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Abstract

ØRTENGREN, N. A. and K. RONALD. *Aspects of temperature regulation in harp seal pups evaluated by in vivo experiments and computer simulations.* Acta physiol. scand. 1978. 103: 263-269.

Temperature regulation in harp seal pups (*Pagophilus groenlandicus*) has been studied by *in vivo* measures of oxygen consumption, body temperatures and *in vivo* determinations of the thermal values of pups. The experimental animals were restrained and exposed to natural weather conditions. Solar loads above 600 W/m² (global radiation) caused hyperthermia. Computer simulations based on the all slopes of the fur indicated that heat dissipation from presently the fur is prerequisite for avoiding hyperthermia under natural weather conditions. Further simulations showed that the lower the temperature of the sea-born harp seal pup, its standard metabolism is only -1°C. While it is used to -39°C as the pup grows, a 10 cm thick layer of blubber and the metabolism rises to 1.5 times standard. High metabolism and tolerance to low deep body temperatures (35°C) is prerequisite for thermal balance in the harp seal pup under cold conditions.

Key words: Metabolism, body temperatures, and speed, solar radiation, for simulation, thermal balance model.

When the harp seal (*Pagophilus groenlandicus*) hauls up on drifting ice to give birth to white-furred pups, 2 or 4 weeks after birth the "whitecoats" grow from 7 to about 35 kg solely by deposition of subcutaneous layers of blubber up to 10 cm thick. The white fur at 23 mm thick, is shed 2 to 4 weeks after birth (Severtsen 1941; Friach and Ørtengren 1978).

At birth the seal is exposed to and copes with, a variable and often harsh thermal environment. The seal's thermoregulatory mechanisms have been investigated by only few others. Davydov and Makarova (1965) found that the heat production of the newborn doubled from 73 to 145 W with immersion in ice water while there would be only small metabolic response to cooling at 2 to 4 weeks of age. A high metabolic response to cold air has been reported for moulting whitecoated harp seals (Iversen and Krog 1973).

The thermal conductance of the fur has been reported to be 1.9 to 2.8 W/m² °C still air and increasing with 0.05 times the square power of the wind speed (Ford & Ørisland 1968; Ørisland *et al.* 1978). Gray *et al.* 1974 reported the presence of high amounts of brown fat in the blubber layers of the newborn (7 to 14 kg) harp seal. Ørisland and Ronald (1973) found that solar radiation has a profound heating effect on the skin of whitecoats lying on the ice and suggested that critical air temperature determined in the laboratory can not be used to predict metabolism and behaviour under field conditions.

Thus a good understanding of several aspects of the thermal adaptations of the harp seal pups has been reached. However, an integrated quantitative description of heat loss under natural weather conditions still is needed both for predictions of survival capacity of this species and because of the general need for heat balance models in ecological analysis.

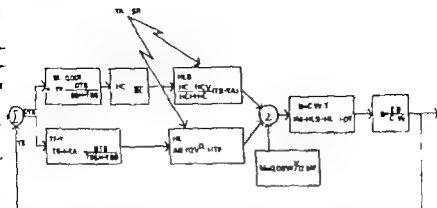
Towards producing such a model metabolism and body temperatures were measured in 7 whitecoated seals exposed to natural weather conditions and the effect of solar radiation on the heat transfer through a fur sample was determined. A thermal simulation model encompassing the conventional ways of describing metabolism, tissue and fur insulation, body surface areas and dry weather conditions was programmed in APL (Iverson 1962). The model was used to evaluate the thermoregulatory capacity of the harp seal pup.

Materials and methods

Harp seal pups were transported from the ice off the Magdalen Islands, Canada, 41–22 h prior to experiments. During measurement the experimental animal was secured to a plywood board by means of aluminium hoops. A respiration valve was mounted close to the seal's nostrils and sealed off with a rubber respiration mask firmly covering the head. A Technology Versatronics OCC1000 continuous oxygen consumption computer was calibrated with a Hofmann-Michaelis dry gas meter used in conjunction with a Scholander 0.5 ml analyzer (Cisolazio *et al.* 1963). The oxygen computer agreed within 5% with the gasmeter—0.5 ml system. Temperatures were recorded with a thermistor probe inserted about 30 cm into the rectum and by a thermistor needle held parallel to the skin surface. Thermal properties of the fur sample exposed to solar radiation were determined using a heat flow apparatus as described by Ørisland and Lavigne (1976). An Eppley solarimeter was placed level to the fur. Solar irradiance was varied by alternately shading off and exposing the fur sample to a given set of air temperature and wind speed through natural variations in solar irradiance.

A simulation model based mostly on conventional thermal physiological parameters was programmed in APL according to the control flow diagram given in Fig. 1. The transfer functions used are presented in the diagram, and the variables are as follows:

TB	= deep body temperature	°C
TBS	= set point and lower limit of regulation band for deep body temperature	°C
TBSH	= upper value for regulation band	°C
DTB	= error signal (DTB = TB - TBS)	°C
TA	= air temperature	°C
V	= wind speed	m s ⁻¹
BR	= solar irradiance (global)	W m ⁻²
HCI	= heat transfer coefficient peripheral tissues (HCI = KF/BL)	W m ⁻² °C ⁻¹
KF	= heat conductivity of fat (0.205 See Carlson and Halsey 1970)	W m ⁻¹ °C ⁻¹
BL	= "functional" blubber thickness	m
AB	= body surface area excluding the flippers	m ²
HCV	= heat transfer coefficient of the fur (HCV = 1.9 + 0.05 V ²)	W m ⁻² °C ⁻¹



1 Control flow diagram for regulation of heat balance of harp seal pups. Deep body temperature compared with the reference temperature (TBS) and the error signal (DTB) is used to determine metabolic blubber thickness (BL) and the flipper surface temperature (TF). Heat loss from the body (HLB) is based on the blubber and fat insulation being coupled in series. Also the heat loss from the flipper (HL) is considered in parallel HLB. Heat production (H) is not coupled to the controller. The difference between heat loss and heat production determines the energy content (EB) of the body and thus deep body temperature. Disturbing elements are air temperature (TA), wind speed (V) and solar radiation (SR). The effect of solar radiation is to decrease the heat loss from the body (calculations are indicated in the figure) by a factor of 0.18 for 45% of the body surface excluding the flippers.

Flipper surface temperature	°C
Flipper surface area	m ²
metabolism	W
heat loss from flippers	W m ⁻²
heat loss from body	W m ⁻²
time between calculated values of TB	
body weight	kg
specific heat capacity of body	J kg ⁻¹ °C ⁻¹
energy (heat) content of body	J

The insulation model is based on proportional control of blubber/skin heat transfer coefficient and flipper temperatures and heat flows are calculated for plane surfaces and layers.

Results

Air temperatures ranged between -2 to 23°C, wind speeds 0.1 to 5 m/s and solar irradiance (global radiation) ranged up to 800 W m⁻² during the *in vivo* experiments. No significant metabolic response to changes in heat load was found. However deep body temperatures varied between 35°C and 41°C dependent on weather conditions. Low deep body temperatures are found in newborn (7-12 kg) pups both during experiments and when kept for about 12 h in an outside enclosure at air temperatures around 0°C, wind speed 2 m s⁻¹ and cloudy rainy and sleeting weather. High body temperatures were caused by solar irradiance above 600 W m⁻². Such solar irradiance gave skin temperatures from 38 to 41°C on the exposed side of the body. The metabolism was 1.5 ± 0.32 (S.D.) times higher than that could be expected from Kleiber's formula (Kleiber 1961). A summary of the experiments is presented in Table 1.

TABLE 1 Summary of measurements of metabolism in harp seal pups exposed to natural weather conditions. Metabolism were read with about 10 min intervals and values are calculated for U_{10} per 1 O_2 STP

Seal No	Weight kg	Mean metabolism W (S.D.)	Duration of experiment min	Windspeed m s^{-1}	Environmental conditions	
					Air temperature $^{\circ}\text{C}$	Solar radiation W m^{-2}
1	8.3	24.5 ± 5.5	529	0-7	7 to 23	0
2	10.5	24.7 ± 1.9	220	1-2	1 to 2	20-422
3	10.5	24.6 ± 1.9	490	0.5-3	-2 to 2.5	<20
4	9	27.2 ± 3.3	493	4-5	-2 to 1	29-770
5	19	67.4 ± 20.8	170	1-2	-1.5 to 1.5	30-100
6	20	49.0 ± 8.9	290	0-3	1.5 to 9	90-700
7	20	42.9 ± 7.8	257	0.5	-2 to 0	90-700

The ratio U also named the solar utilization factor is the heat load (at skin level) to solar radiation divided by the solar irradiance (Ørissland 1971). In 8 experiments solar irradiance was changed by alternate shadowing and exposure of the fur sample with constant wind speed and air temperatures. It was found to be 0.19 ± 0.09 (S.D.). For 5 expts the heat loss decrement due to solar radiation was calculated as the difference between expected and actual heat loss during continuous exposure of the fur sample. The expected heat loss was the heat transfer coefficient (Ørissland *et al.* 1977) multiplied by the skin to air temperature gradient. This resulted in a utilization factor (U) of 0.19 ± 0.09 . Multiple regression analysis indicated that only 10 per cent of the variation in U was due to variations in wind speed and air temperature.

The average difference between simulated and *in vivo* deep body temperatures was less than 0.5 ± 0.7 (S.D.) $^{\circ}\text{C}$ for simulations of the separate expts. Such a simulation fidelity was obtained by using reference temperatures between 36.5 and 37.5°C and metabolism between 1.2 and 2 times standard (Kleiber).

Simulation of deep body temperature of a 20 kg whitecoat (seal no. 6) with specific heat capacity $3.100 \text{ kg}^{-1} \text{ }^{\circ}\text{C}^{-1}$ and a constant metabolism 2 times what could be expected from Kleiber's formula is presented together with *in vivo* values in Fig. 2.

Simulation of a small, lean whitecoat (10 kg and 1 cm of blubber) with standard metabolism resulted in a lower critical temperature (at wind speed 0.2 m/s) of $-1 \pm 1^{\circ}\text{C}$ while with a blubber thickness of 10 cm the critical air temperature was $-23 \pm 1^{\circ}\text{C}$. Simulating a large and lean (20 kg and 1 cm blubber) whitecoat having standard metabolism, resulted in critical air temperature of $-3 \pm 1^{\circ}\text{C}$, while increasing the blubber thickness to 10 cm gave a lower critical air temperature of $-27 \pm 1^{\circ}\text{C}$. For metabolism 1.5 times standard, the critical air temperature was $-59 \pm 1^{\circ}\text{C}$ for the 20 kg whitecoat with 10 cm blubber.

Also by simulation the whitecoats would rapidly become hyperthermic if the heat loss through the flippers was set to zero. For a 20 kg whitecoat the upper critical temperature for 1.5 times standard metabolism without heat loss through the flippers, was only $-21 \pm 1^{\circ}\text{C}$. Intact flippers allowed maintenance of constant deep body temperatures at air temperatures up to $15 \pm 1^{\circ}\text{C}$. Heat dissipation through flippers was also necessary for the simulated harp seal pup to survive solar radiation below 600 W m^{-2} .

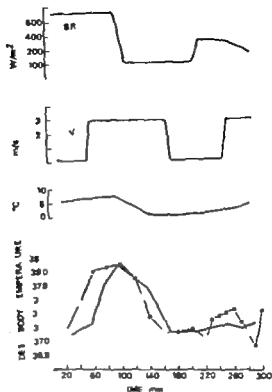


Fig. 1. Simulated (●) and in vivo (○) deep body temperatures of 28 kg harp seal pup in relation to varying levels of solar radiation and wind speed (V) and air temperatures.

Discussion

The present variability and range in deep body temperatures is wider than reported earlier (e.g.) while skin temperatures were in agreement to the values found on free whitecoats on the ice (Ortengren and Ronald 1973). A similar variability in deep body temperatures has also been found by others working on unrestrained northern fur seal pups (Blix pers. comm.).

In a hooded seal weighing approximately 200 kg left to cool for 65 h post mortem the axial cooling, measured about 50 cm from the tail, was 0.29 °C/h, while in another central site close to the largest body diameter (about 1.2 m anterior to the tail) the cooling rate was 0.22 °C/h at air temperature 8 to 1 °C (Ortengren unpublished).

Thus a higher heat loss coefficient or surface to heat capacity ratio is present for the anterior as compared to the mid part of the body. Consequently the mid part can be expected to show more stable temperatures. Also, on the basis of the rapid shifts in blood circulation recorded in conjunction with diving reflexes (Blix 1976), resulting in cooling and heating of peripheral tissues, it is possible that the animals are able to adjust their insulation and deep body temperatures rapidly. Further studies encompassing simultaneous measurements of rectal and other locations for deep body temperatures are necessary in order to elucidate this hypothesis.

TABLE 1 Summary of measurements of metabolism in harp seal pups exposed to actual weather conditions. Metabolism was read with about 10 min intervals and values are calculated in (l) per l O₂ STP

Seal No	Weight kg	Mean metabolism W (S.D.)	Duration of experiment min	Windspeed m/s	Environmental conditions	
					Air temperature °C	Solar radiation W/m ²
1	8.3	24.5 ± 5.5	529	0-7	7 to 23	0
2	10.5	24.7 ± 1.9	220	1.2	1 to 2	20-422
3	10.5	24.6 ± 1.9	490	0.5-3	-2 to 2.5	20
4	9	27.2 ± 3.3	493	4-5	-2 to 1	29-778
5	19	67.2 ± 70.8	170	1.2	-1.5 to 1.5	30-188
6	20	49.0 ± 8.9	290	0-3	1.5 to 9	30-708
7	20	42.9 ± 7.8	557	0.5	-2 to 8	98-740

The ratio U also named the solar utilization factor is the heat load (at skin level) to solar radiation divided by the solar irradiance (Ørtisland 1971). In 8 experiments solar irradiance was changed by alternate shadowing and exposure of the fur sample with constant wind speed and air temperatures. It was found to be 0.19 ± 0.09 (S.D.). For 5 expts. the heat loss decrement due to solar radiation was calculated as the difference between expected and actual heat loss during continuous exposure of the fur sample. The expected heat loss was the heat transfer coefficient (Ørtisland *et al.* 1977) multiplied by the skin to air temperature gradient. This resulted in a utilization factor (U) of 0.18 ± 0.09 . Multiple regression analysis indicated that only 10 per cent of the variation in U was due to variations in wind speed and air temperature.

The average difference between simulated and *in vivo* deep body temperatures was less than 0.5 ± 0.7 (S.D.) °C for simulations of the separate expts. Such a simulation fidelity was obtained by using reference temperatures between 36.5 and 37.5°C and metabolism between 1.2 and 2 times standard (Kleiber).

Simulation of deep body temperature of a 20 kg whitecoat (seal no. 6) with specific heat capacity $3100 \text{ kg}^{-1} \text{ } ^\circ\text{C}^{-1}$ and a constant metabolism 2 times what could be expected from Kleiber's formula is presented together with *in vivo* values in Fig. 2.

Simulation of a small, lean whitecoat (10 kg and 1 cm of blubber) with standard metabolism resulted in a lower critical temperature (at wind speed 0.2 m/s) of $-1 \pm 1^\circ\text{C}$ while with a blubber thickness of 10 cm the critical air temperature was $-23 \pm 1^\circ\text{C}$. Simulating a large and lean (20 kg and 1 cm blubber) whitecoat having standard metabolism, resulted in critical air temperature of $-3 \pm 1^\circ\text{C}$, while increasing the blubber thickness to 10 cm gave a lower critical air temperature of $-27 \pm 1^\circ\text{C}$. For metabolism 1.5 times standard, the critical air temperature was $-59 \pm 1^\circ\text{C}$ for the 20 kg whitecoat with 10 cm blubber.

Also by simulation the whitecoats would rapidly become hyperthermic if the heat loss through the flippers was set to zero. For a 20 kg whitecoat the upper critical temperature for 1.5 times standard metabolism, without heat loss through the flippers, was only $-21 \pm 1^\circ\text{C}$. Intact flippers allowed maintenance of constant deep body temperatures at air temperatures up to $15 \pm 1^\circ\text{C}$. Heat dissipation through flippers was also necessary for the simulated harp seal pup to survive solar radiation below 600 W/m^2 .

to direct sunshine, the pup seems incapable of reducing the metabolism sufficient to prevent overheating, and therefore depends on heat dissipation from primarily the head and to some extent the skin in general.

The balance model has been produced and can be used as demonstrated above in a series of physiological responses to changing weather conditions. In addition the model probably be useful in future assessments of energy balance of other marine mammals.

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Significant variability in deep body temperature also has been noted in small mammals and birds and can partially be explained by changes in the reference or preferred body temperature (Morhardt 1975). Such changes are indicated by the present work as temperatures varying from 36.5 to 37.5 were necessary to obtain acceptable simulations of the experiments.

The simulations employed a specific heat capacity of $3.100 \text{ J/kg } ^\circ\text{C}^{-1}$ corresponding to a 0.23 to 0.77 ratio of fat to lean body mass with specific heat capacities of 1.88 and $3.470 \text{ J/kg } ^\circ\text{C}^{-1}$ respectively (Burton 1935; Minard 1970). Such a body composition is acceptable (Silverstein 1941) and it thus appears that treating the body of the harp seal pup as a composite of plane surfaces and layers related to the 2/3 power of the body weight (Irving *et al.* 1935; Iversen and Krog 1973) is valid in thermal simulations. While more experiments remain to be performed the present work and computer simulations reflect quantitatively the thermal significance of the blubber and supports the suggestion of Ortisland (1968) that there is a significant circulation through these tissues in response to heat stress.

Wide thermoregulatory variations in blood circulation in the flippers is suggested by the present work to act not only towards maintaining deep body temperature in the cold but for avoiding hyperthermia, the latter because of the limited heat loss that can be caused by circulation of the blubber. Also the present work supports the suggestion of Ortisland and Ronald (1973) that critical air temperatures determined in the laboratory are not sufficient to describe mammalian heat balance under field conditions and appropriate heat load indices (e.g. Ortisland 1974) have been previously established.

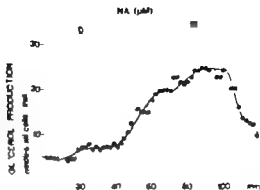
The present metabolism values averaging 1.5 times standard are in close agreement with those reported earlier as reviewed by Irving (1972). In contrast to results published previously (Davydov and Makarova 1964; Iversen and Krog 1973) a metabolic response to cold was not found. However the metabolic values reported by the above authors occurred in response to more severe cooling (immersion in ice water; Davydov and Makarova) than in the present experiments, or was obtained from mouthing pups (Iversen and Krog) with presumably impaired insulation. Actually Frisch and Ortisland (1968) predicted that a 70°C drop in air temperature may cause a doubling of metabolism in the harp seal pup if peripheral circulation is not reduced when the white fur is shed. Inasmuch the variance of the metabolic values obtained here may obscure an eventual metabolic response, the question about whether or not there is a direct temperature related control of metabolism remains unsolved. An arrangement with less restraint of the experimental animal seems necessary in future examinations of metabolic responses to cold.

The simulations revealed, however, that a high metabolic rate is a prerequisite for thermal homeostasis even for the largest and fattest whitecoat and that most animal changes in the thermal environment can be compensated for by metabolic adjustments well inside the range recorded here.

In conclusion the thermal survival of the harp seal pup seems to be accomplished through a combination of high metabolism, most likely accomplished by non shivering thermogenesis in brown fat and muscle (Grav and Blix 1976) and tolerance to low body temperatures under cold conditions. Under warm environmental conditions, especially when

E.
C.

A typical experiment showing the dose-dependent increase in glycerol outflow from fat cells stimulated with 0.1-10 μ M noradrenaline (NA). 10 μ M NA produced a response about 1 μ M in two out of three experiments.



Methods and materials

Myristic fat was obtained from decapitated male Sprague-Dawley rats (Anticommex) weighing approximately 200 g. Isolated fat cells were prepared according to Rodbell (1964) using 2 mg/ml of crude bacterial lipase (Wardington Biochem. Co.). After the final washing, 1 ml aliquots of packed cells was transferred to temperature-controlled plastic chamber containing 2.36 ml Krebs-Ringer phosphate buffer 7.4 with half the recommended calcium concentration, 5.5 mM glucose and 1 bovine serum albumin (Boehringer-Mann, St. Louis). The floating fat cells are subsequently "perfused" (Allen *et al.* 1973b) with this buffer at 37°C and rate of 2 ml/min. Drugs were infused in side-arm before the chamber or in the end of perfusion line, dissolved in the perfusion buffer. Glycerol was measured by method of Lazzari and Tibbling (1964), but the $ZnSO_4$ - $Sn(OH)_2$ precipitation step was eliminated to save the sensitivity. Standard curves for glycerol in the perfusion buffer gave expected values despite modifications. Theophylline and adenosine deaminase (Boehringer, Mannheim), both as analyzed or as used, did not influence the buffer blanks always included in the assay.

Results

There was a basal outflow of glycerol from the isolated perfused fat cells of 4.0 ± 0.5 nmol cells⁻¹ min⁻¹ (n = 31). Lipolysis was stimulated in dose-dependent manner by noradrenaline (Fig. 1) and theophylline (Fig. 2). The combination of noradrenaline and theophylline resulted in additive effects preferentially when submaximally effective concentrations of noradrenaline were used (Table I).

Adenosine deaminase (0.1-1 μ g/ml) was an ineffective lipolytic agent in 3 expts. with perfused fat cells. Thus, adenosine deaminase increased glycerol outflow by only 1.1 ± 0.4 nmol ml cells⁻¹ min⁻¹ whereas 1 μ M noradrenaline in the same cells caused an increase of 23 ± 5.3 nmol ml cells⁻¹ min⁻¹. The enzyme did not increase lipolysis in fat cells stimulated by a submaximally effective concentration of noradrenaline either. Adenosine inhibited lipolysis in perfused fat cells stimulated by noradrenaline or by theophylline. A typical experiment is shown in Fig. 3. The data summarized in Table II show that adenosine antagonizes theophylline-induced lipolysis more effectively than that induced by noradrenaline.

Discussion

The present experiments demonstrate antilipolytic effects of adenosine in micromolar concentrations in perfused fat cells stimulated by noradrenaline or theophylline. The failure

Antilipolytic effect of adenosine in isolated perfused fat cells

By

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Received 15 December 1977

Abstract

HJERMDAHL, P. and A. SOLLEVI. Antilipolytic effect of adenosine in isolated perfused fat cells. *Acta physiol. scand.* 1978. 103. 270-274.

Adenosine markedly inhibits cyclic AMP accumulation in isolated fat cells. Inhibitory effect of adenosine on lipolysis has been difficult to demonstrate. The present study has been performed on isolated "perfused" fat cells where continuous monitoring of the lipolytic rate is possible and where modulating substances, such as adenosine, are not allowed to accumulate. Adenosine deaminase is ineffective as lipolytic agent in perfused fat cells, suggesting no important background activity of adenosine in this system. Micromolar concentrations of adenosine inhibited lipolysis induced by noradrenaline (0.3-1 μ M) and theophylline (1 mM). Theophylline was an effective lipolytic agent also in perfused fat cells, suggesting that antagonism of adenosine is not the major mode of action of this drug on fat cells.

Adenosine, which is formed by the degradation of ATP, may be an important modulator of lipolysis in adipose tissue (cf. Schwabe *et al.* 1975). This proposition is based on the findings that adenosine is released from fat cells *in vitro* (Schwabe *et al.* 1973) and that adenosine and analogues of adenosine are potent inhibitors of cyclic AMP accumulation in isolated fat cells (Fain *et al.* 1972, Ebert and Schwabe 1973, Stock and Prilop 1974, Hjerm Dahl and Fredholm 1976). There are, however, conflicting results as to the effect of adenosine on lipolysis in isolated fat cells. Thus, Ebert and Schwabe (1973) found that adenosine inhibited lipolysis, whereas other studies (Fain *et al.* 1972, Fain 1973, Stock and Prilop 1974, Hjerm Dahl and Fredholm 1976) have failed to reveal an antilipolytic action of the cyclic AMP response of the fat cells. This apparent lack of antilipolytic effect of adenosine might be due to a high background activity of adenosine in conventional fat cell incubates. We have therefore studied the effect of adenosine on lipolysis in "perfused" fat cells (Allen *et al.* 1973 b), a system which prevents accumulation of modulating substances such as adenosine in the vicinity of the fat cells. We have also used this experimental model to examine the effect of adenosine deaminase and theophylline, two drugs which may induce lipolysis by antagonism of adenosine (Schwabe and Evert 1974) in a situation where a low concentration of adenosine should prevail.

- 10 The influence of adenosine (1 10 μ M) on lipolysis in perfused fat cells stimulated by noradrenaline or by theophylline. Note that theophylline-stimulated lipolysis is more sensitive to adenosine than noradrenaline-stimulated lipolysis.

Stimulating agent	Net glycerol production rate (nmol min^{-1} ml cells $^{-1}$)	% inhibition by adenosine	
		1 μ M	10 μ M
1 noradrenaline	22 \pm 9	29 \pm 6 (3) ^a	—
1 noradrenaline	36 \pm 12	25 \pm 7 (-3) ^a	34 \pm 7 (3)
1 theophylline	24 \pm 5	39 \pm 5 (4) ^a	78 \pm 5 (4) ^a

805. ^a $p < 0.01$ ^a $p < 0.001$

is of adenosine deaminase are due to removal of adenosine, since unspecific effects of cytosine should have shown up also in perfused fat cells.

The lipolytic effect of theophylline does not appear to be linked to its ability to inhibit cyclic AMP breakdown by phosphodiesterase (Schwabe *et al* 1972, Allen *et al* 1973 a) and, it has been proposed that theophylline induces lipolysis by antagonizing effects of cellular adenosine (Ebert and Schwabe 1973, Schwabe and Ebert 1974). This suggestion is based on findings that theophylline inhibits effects of adenosine in many organs and that theophylline and adenosine deaminase produce similar effects in conventionally incubated cells.

The present experiments show that adenosine is a more potent antagonist of lipolysis induced by theophylline than of that induced by noradrenaline. The potency of adenosine may however be inversely related to the cyclic AMP levels of the fat cells (Fain 1973, Ulfhake 1977). Therefore, the greater sensitivity of theophylline-induced lipolysis could be explained by the fact that theophylline increases cyclic AMP considerably less than does noradrenaline. The dissociation between the effects of theophylline and adenosine deaminase observed in the present expts. with perfused fat cells is not easily reconciled with the above mentioned idea that the major mode of action of theophylline is through antagonism of phosphodiesterase. The present findings do not rule out the possibility that adenosine and theophylline exert their effects on fat cells through a common mechanism, although they emphasize the need for further proof.

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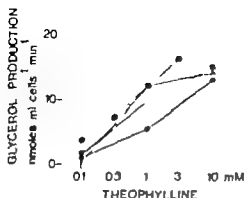


Fig. 2

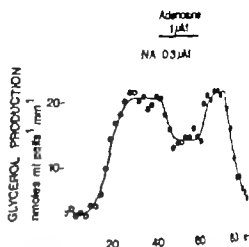


Fig. 3

Fig. 2. Dose-dependent glycerol production rate in four experiments with perfused fat cells stimulated by 0.1-10 mM theophylline. The experimental design was the same as that illustrated in Fig. 1.

Fig. 3. A typical experiment demonstrating the antilipolytic effect of 1 μ M adenosine in perfused fat cells stimulated by 0.3 μ M noradrenaline (NA).

In several previous studies to demonstrate antilipolytic effects of adenosine in concentrations which inhibit cyclic AMP accumulation in the fat cells markedly (Fain *et al.* 1973; Fain 1973; Stock and Prilop 1974; Hjerdahl and Fredholm 1976) is probably due to accumulation of adenosine in the incubates to such an extent that maximal antilipolytic effects of adenosine already prevailed.

The technique of "perfusing" isolated fat cells (Allen *et al.* 1973b) was chosen in the present study because of the possibility of studying the effects of adenosine without the interference of a high background activity of adenosine, which accumulates rapidly in conventional fat cell incubates (Schwabe *et al.* 1973). The failure to demonstrate lipolytic effects in perfused fat cells of adenosine deaminase, which induces lipolysis in conventional incubated fat cells (Fain 1973; Schwabe and Ebert 1974; Hjerdahl and Fredholm 1976) may be taken as evidence that the perfusion technique reduces the adenosine concentration at the fat cell to unimportant levels. These results also support the contention that the

TABLE 1 The effect of theophylline (Theo) on lipolysis in perfused fat cells stimulated by noradrenaline (NA, 0.1-3 μ M) in 6 separate expts. Note that theophylline caused further decrease in glycerol production rate when submaximally effective concentrations of noradrenaline were used.

NA (μ M)	Theo (mM)	Glycerol production rate (nmol ml cells ⁻¹ min ⁻¹) during		
		NA	NA + Theo	
0.1	0.1	46.4	72.5	(+56%)
0.3	0.3	6.2	41.6	(+59%)
1.0	1.0	18.8	23.1	(+23%)
1.0	1.0	56.7	71.5	(+26%)
1.0	3.0	29.8	27.8	(-7%)
1.0	3.0	40.6	46.3	(+14%)

Intra-abdominal pressure changes during natural movements in man

By

STEIN GRILLNER, JOHNNY NILSSON and ALF THORSTENSSON

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Abstract

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Weight of the upper part of the trunk is partially transmitted to the pelvis as the vertebral column muscles and the abdominal cavity are contracted. High pressure can be generated within the abdominal cavity. The abdominal spaces can then transmit part of the weight to the upper part of the body. Intra-abdominal pressure recordings have been performed during locomotion and other natural movements with intragastric pressure recordings. With each step, there is a phasic variation in intra-abdominal pressure. The peak value of the peak vertical force exerted by the leg against the ground. The peak values increase progressively with the speed of walking/running up to a mean of 16 mmHg and the trough values of 16 mmHg. The phasic variation in each step is due to phasic variation of the abdominal muscles, with an EMG activity starting 50 ms or more before foot contact. If a load is put on the back, the pressure changes and at the highest speed of running the pressure is significantly higher than without this additional load. After a jump down from moderate height 24 mmHg increase in pressure and can often exceed 100 mmHg. These pressure changes are presumably act to unload the spine under the prevailing biomechanical conditions and, in fact, there will be an effect on the circulatory system.

Extremely little detailed information is available on how the trunk is controlled during different types of movements, despite the fact that it represents the larger part of the body weight, and consequently is of paramount importance in the equilibrium control during locomotion and posture. One special aspect relates to the control of the pressure within the abdominal cavity.

The load of the upper part of the body in man would entirely be transmitted to the vertebral column if the intraabdominal pressure (IAP) was zero. However, if a pressure is created in the abdominal cavity by the joint action of the muscles of the pelvic floor, the abdominal wall, and the diaphragm, this pressure can act to support the upper part of the body and thereby unload the spine to a degree proportional to the actual pressure (cf. Bartelink 1957, Morris *et al.* 1961, Elk and Webb 1962, Davis and Troup 1964). Indeed, pressure changes above 200 mm Hg have been recorded during lifting (Morris *et al.* 1961, Elk and Webb 1962). The degree of increase in pressure is related to the

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Intra-abdominal pressure changes during natural movements in man

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Abstract

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Most of the upper part of the trunk is partially transmitted to the pelvis as the vertebral column, muscle walls around the abdominal cavity are contracted. High pressure can be generated within the trunk (200 mmHg). The abdominal space can then transmit part of the weight to the lower part of the body. Intra-abdominal pressure recordings have been performed during locomotion and other natural movements as intragastric pressure recordings. With each step there is a phasic variation in pressure, with the peak coinciding with that of the peak vertical force exerted by the leg against the ground. The peak values increase progressively with the speed of walking, rising up to 100 mmHg during running and with trough values of 16 mmHg. The phasic variations in each step is due to phasic changes in the intra-abdominal pressure, with an EMG activity starting 50 ms or more before foot contact. If extra load is put on the back, the pressure changes and at the highest speed of running the pressure can be significantly higher than without the additional load. After jumping down from standing height 2.5 m, the average pressure is 89 mmHg and can often exceed 100 mmHg. These pressure changes are thought to be important in relation to the spine under the prevailing biomechanical conditions and, in some cases, there will be an effect on the circulatory system.

Surprisingly little detailed information is available on how the trunk is controlled during different types of movements, despite the fact that it represents the larger part of the body weight, and consequently is of paramount importance in the equilibrium control during locomotion and posture. One special aspect relates to the control of the pressure within the abdominal cavity.

The load of the upper part of the body in man would entirely be transmitted to the pelvic girdle by the vertebral column if the intraabdominal pressure (IAP) was zero. However, if a pressure is created in the abdominal cavity by the joint action of the muscles of the pelvic floor, the abdominal wall, and the diaphragm, this pressure can act to support the upper part of the body and thereby unload the spine in a degree proportional to the actual pressure (e.g. Bartelink 1957, Morris *et al.* 1961, Elie and Wehn 1962, Davis and Troup 1964). Indeed, pressure changes above 700 mmHg have been recorded during lifting (Morris *et al.* 1961, Elie and Wehn 1962). The degree of increase in pressure is related to the

weight lifted and to the horizontal distance from the load to the spine (Davis and Jay 1964, Asmussen and Poulsen 1968, Andersson *et al.* 1976). These pressure increases are primarily depending on active contractions of the oblique and the transverse abdominal muscles rather than the rectus abdominis (Bartelink 1957, Morris *et al.* 1961). The degree of unloading of the spine is not clear but has been estimated to be 30–40% when lifting heavy weights (Morris *et al.* 1961, Eke and Wehn 1962).

Except for Eke's (1966) data on skiing (ski-jumping (1)), nothing is known about the control of the intraabdominal pressure during normal activities such as walking, running, walking with a back pack or jumping down a few tenths of a meter. In the present paper we have studied such natural motor activities. The specific questions we wanted to explore were

1. What changes occur in IAP during locomotion?
2. Does an extra load on the back influence the magnitude of the IAP during locomotion?
3. How are the changes in IAP related to the vertical force produced in each step or jump?
4. Are the changes in IAP related to active contractions of the muscles of the abdominal wall?
5. How is the EMG activity in the abdominal muscles related in time with the locomotion? Is IAP and the rise in vertical force during locomotion and jumping (i.e. is the development of IAP a pre-programmed process)?

Some of these results have been reported in preliminary form (Thorstenson *et al.* 1977).

Material Methods and Procedure

Subjects

A total of 13 subjects, 11 males and one female, participated in the study. Their mean age, height and weight were 25 yrs (range 18–34 yrs), 180 cm (range 173–187 cm) and 74 kg (range 65–87.6 kg), respectively. All were "normal" subjects, habitually active but not involved in regular training. None of the subjects had a history of back pain.

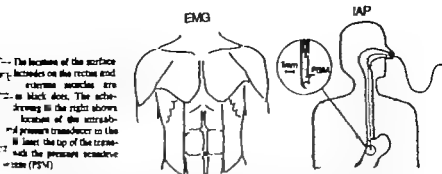
Methods

The intraabdominal pressure (IAP) was measured intragastrically with a tip pressure transducer (Micro-tip, PC 340 Millar Instr. Houston, Texas, USA) designed for pressure recordings retrogradely. The transducer had a frequency response of DC to 70 kHz and a normal accuracy of ± 0.5 mmHg. The selected range from -300 to +400 mmHg. Compared to the balloon that has often been used before for IAP recordings (e.g. Bartelink 1957, Morris *et al.* 1961, Davis and Troup 1964, Eke and Wehn 1962, Asmussen and Poulsen 1968), the use of a transducer placed in the entrance allows recordings of rapid pressure changes without any associated damping effects.

The transducer catheter was placed in a flexible plastic tube ($\varnothing = 3.5$ mm) and introduced through the nose after light local anaesthesia (Fig. 1). The position of the transducer in the ventricle was easily verified by the behaviour of the pressure during deep breathing and Valsalva manoeuvres. In the entrance the pressure sensitive membrane placed on the side of the catheter was allowed to protrude 2–3 mm from the end of the plastic tube (Fig. 1). Usually the distance from the nostrils to the transducer was 45–55 cm depending on the height of the subjects.

The control unit (Millar TCB-100, weight 0.6 kg) was carried by the subject in a small case over the sternum. Electrical calibration was built into the control unit.

Control experiments were carried out with the transducer in a plastic or glass bottle carried by the subjects when performing the different exercises. The content of the bottles was varied to simulate different conditions. In no case did the pressure under these artificial conditions exceed 3 mmHg.



The location of the surface electrodes on the rectus abdominis and the obliquus externus muscles according to Fig. 1. The distance between the electrodes is 4 cm. The frequency range is 30 Hz to 10 kHz. The preamplifiers P15; total weight 2.2 kg) are attached to a specially made light-weight backpack frame.

The experiments are performed including sitting and running either on a motor-driven mill (Series A), or over a force platform (Series B). Also running in place and different jumps were included in Series B.

Before the experiments on the treadmill started the subjects got used to the test situation in 2-3 training sessions. On the test day sitting and running are performed at the belt speeds 8, 12, 16, 20 and 24 km h⁻¹. The velocities were applied in the following order: 8, 12, 16, 20 and 24 km h⁻¹. A maximum of 20 consecutive steps of steady locomotion was performed at each velocity. Recovery periods of 1-2 min are allowed between each run. After a longer rest (3-5 min) the protocol was repeated with an extra back load. The female and 3 males weighing less than 70 kg carried 19 kg load and the remainder carried 27 kg. The weight consisted of 5 iron bars 11 kg each that were symmetrically placed close to the back on the frame mentioned above. The mean IAP as well as the peak and the trough (min. IAP) values are recorded and related to the speed of locomotion and to the step sequence. The different phases of the step cycle are assessed by accelerometers placed over the knee joints, and in some experiments by microswitches mounted in the sole of conventional sport shoes used as test shoes.

In this series different motor behaviours were performed on a force platform (Type A, Kistler Inc., AG, Winterthur, Switzerland), mounted level with the floor. The movement of the force platform was loaded as negligible. The platform allowed measurements of 3 perpendicular force components. In the present study however only vertical force (the predominating force component) was used together with IAP and EMG.

The speed of sitting and running over the force platform was adjusted to correspond to approximately 8 and 16 km h⁻¹ respectively. The speed of locomotion is assessed by measuring the duration of the foot-plate contact and compared with that obtained when sitting and running on the treadmill with different speeds. The frequency of running and jumping in place is determined by metronomes. The jumps down to the platform were performed from 40 cm high platforms. At least 10 steps or jumps were recorded in each experimental situation.

The IAP and the EMG were recorded simultaneously with the signals from the electrogoniometers and accelerometers (Series A) or the vertical force (Series B) on an 8 channel recorder (Mangoruf 803, Elema, Stockholm, Sweden), with a straight frequency response from 0 to 1200 Hz.

Results

Walking and running

IAP in relation to speed. The IAP recordings during walking and running showed phasic changes. High and low pressures alternated in a regular fashion. The peak IAP demonstrated a steep, almost linear increase with increased speed both for walking and running.

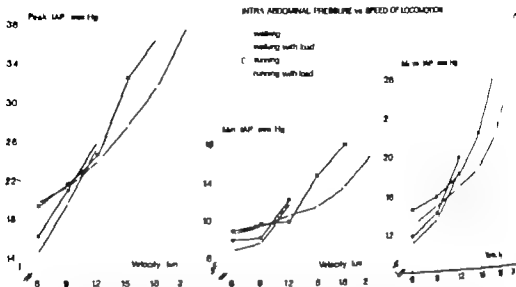


Fig. 2. The intraabdominal pressure (IAP) in relation to the speed of walking and running as a model. The peak and the trough (min. IAP) values of the phasic IAP changes are shown together with the mean IAP calculated from the area under the pressure curve. The filled symbols represent IAP during walking and running with an extra back load of 18–7 kg. Values are means for 10 subjects and of 10 consecutive steps for each subject. The standard deviations, for practical reasons not included in the figure, were 3–7 mmHg.

(Fig. 2 left) reaching values around 38 mmHg at the highest running velocity. Also the mean IAP and consequently the mean IAP values, increased with higher speeds of locomotion (Fig. 2 middle and right). The increase in min IAP was, however, not as steep as for peak IAP which resulted in an increase in the peak to peak values with speed, from about 8 mmHg at slow walking to 22 mmHg at the highest speed of running. It is noteworthy that during running a relatively high IAP is also maintained between the peak values. At the lowest velocity the peak IAP was higher for running than for walking. This difference was not present when walking and running were compared at higher velocities (Fig. 2).

The effect of an extra back load on IAP The effect on the IAP of the symmetrically placed extra back load is shown in Fig. 2. The extra load caused a significant ($p < 0.05$) increase in IAP only at the highest speeds used (15 and 18 km/h). The behaviour of the IAP in relation to speed of locomotion was otherwise similar with and without load. The fastest speed (21 km/h) was not compared due to difficulties encountered by the subjects when running with the extra load at this velocity.

IAP in relation to vertical force in each step Fig. 3 and 4 show the high degree of correlation seen in the development of IAP and vertical force produced in each running step. The IAP tended to build up somewhat before the instance of foot contact (Fig. 3 left) and was actually leading the development of vertical force except for the initial sharp rise in force (cf. Fig. 3 right).

In walking the situation was more complicated. During the support phase of one leg on the force platform both the vertical force and the IAP showed a biphasic increase. However, due to the occurrence of a double support phase during walking, it was difficult to exactly relate the increase in IAP to either the heel strike of one leg or the push-off of the other.

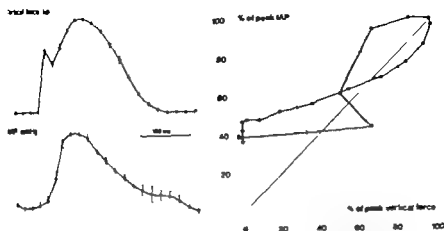


Fig. 3. The intraabdominal pressure in relation to vertical force exerted in one step. Here running over a platform at a speed of approximately 9 km h^{-1} . Values are means ± 1 S.E. for 6 subjects each running 10 runs. To the right the two variables are compared in relative terms. The thin line shows the relationship.

EMG activity in the abdominal muscles. Having established that the changes in IAP are phasic and related to the step cycle it was of interest to see if the increase in IAP was a phasic phenomenon or if it was associated with phasic contractions of the abdominal muscles. Fig. 4 gives a typical example of EMG activity in the abdominal muscles during running, this time performed in place on the platform to obtain force recordings from successive steps. In each step there was a phasic activation of both the rectus abdominis and the oblique muscles. The EMG of both muscles started prior to foot contact and also during the increase in IAP (Fig. 4). Thus it can be concluded that in each step there is a phasic activation of the abdominal muscles. This contraction creates the conditions necessary for the rapid increase in pressure (see discussion). No attempt was made to compare the EMG activity in the rectus abdominis and the oblique muscles on a quantitative basis. However the EMG of the oblique muscles appeared to be more consistently associated in time with the increase in IAP than the EMG of the rectus abdominis. The lateral part of the abdominal wall consists of three layers of muscles (the external oblique, the internal oblique



Fig. 4. Vertical force, intraabdominal pressure and EMG of the right rectus abdominis and oblique muscles during three consecutive steps in one subject running in place on a force platform.

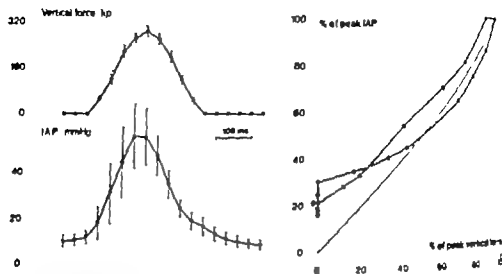


Fig. 5 The intra-abdominal pressure in relation to vertical force exerted when jumping in place on a force platform. Values are means \pm 1 S.E. for 6 subjects each performing 10 jumps. To the right the two variables are compared in relative terms (cf Fig. 3).

and the transverse muscle). The cutaneous recording electrodes will of course pick up the largest signals from the external oblique muscle but will in all likelihood also pick up some activity from the deeper layers. However it is likely that these muscles are weakly coactivated since it would be desirable to have muscle fibres with different orientations activated at the same time.

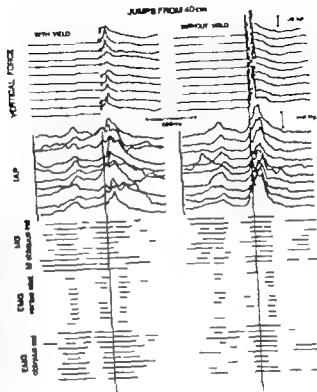
Jumping

Jumping in place Different jumping regimes were performed on the force platform using both one and two legs. A good correlation was always seen between the IAP and the vertical force as shown for two leg jumps in Fig. 5. The covariation between IAP and vertical force was similar also to that seen in running (cf Fig. 3). Compared to running the differences from one individual to the other were somewhat larger in jumping (Fig. 5).

Jumping down from a height The jumps down onto the force platform from a height of 40 cm were done in two different ways, either with a yield in the hips, knee and ankle joints or without yield i.e. hitting the platform with the instruction to keep the joints stiff. The jumps without yield caused the largest peaks in vertical force and IAP (Fig. 6). Peak IAP values exceeding 100 mmHg were often seen (the mean value for the 6 subjects was 89 ± 15 (S.E.) mmHg). The IAP started building up clearly before the instance of foot contact. The increase in IAP was associated with an increase in EMG activity in the abdominal muscles. Again the EMG activity of the oblique muscles was more consistently related to the rise in IAP than that of the rectus abdominis (Fig. 6). The EMG pattern appeared similar in jumping both with and without yield.

Discussion

The results of the present study have demonstrated substantial increases in pressure within the intra-abdominal cavity during common motor activities such as walking.



6. Superimposed recordings of force and intra-abdominal pressure (IAP) in one subject during jumps from 40 cm onto force platform, with (left) and without yield (right) in hip, knee and ankle joints. Vertical lines denote the time of foot-contact. The change of the EMG activity in the abdominal and oblique (lateral) muscles shown below horizontal lines. The first peak in IAP and the corresponding EMG activity coincide with the take-off from the 40 cm platform.

and jumping. The increases in IAP were phasic and occurred when the vertical load on the body was increased. The rise in IAP was associated with increased EMG activity in the muscles of the abdominal wall. It is important to note that the phasic muscle activity started prior to foot contact. It forms clearly a part of the neural control system for locomotion and is integrated into the "locomotor program" (see Grillner 1975).

It is striking how similar the changes in vertical force were to the intra-abdominal pressure recordings (see Fig. 4). This is presumably related to two different things:

(a) that the relevant muscles are activated before and during each contact period (Fig. 4, 6) and (b) that the higher the level of pressure the larger the degree of muscle activity. It is noteworthy, however, that the degree of EMG activity was not following the pressure recordings closely (see Fig. 4).

It is well known that actively contracting muscles produce more tension when extended (e.g. Rack and Westbury 1969). This increase in tension (stiffness in e.g. N mm^{-1}) will inevitably play a role during load compression (e.g. Grillner 1972). The abdominal muscles form the wall of an elongated cavity similar to an elongated ball. In each contact period, the upper part of the body will bounce down and exert an extra pressure on this "ball". Depending on the stiffness of the muscle wall, a large or a small pressure will result within the cavity. The amount of pressure depends on the stiffness, which is related to the degree of muscle contraction. Although the stiffness of the muscles lining the abdominal cavity is unknown, it is clear

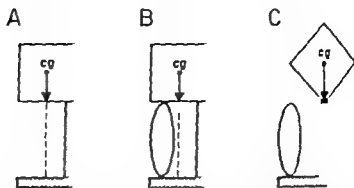


Fig. 7. Schematic representation of how the weight of the upper part of the body may be transmitted to the pelvis. In A only the vertebral column is assumed to contribute. In B the abdominal cavity is assumed to take up part of the weight. In C the vertebral column is again solely responsible, although an intrasideral pressure is built up. cg, abbreviation for center of gravity of the upper part of the body.

that this factor ought to contribute to the building up of pressure. These properties in themselves can then account for the similarity between the IAP and the vertical force.

The rigid structure (cylinder) thus formed by the contracting muscles of the abdominal wall allows a fast rise in IAP in response to sudden increases in vertical force, as e.g. in running or jumping down from a height. Such a mechanism obviates the time delays due to reflexes and electromechanical coupling (Grillner 1972). The latter factor being not too important in the present study considering the rather sluggish system with the wide sheets of oblique and transverse abdominal muscles. This may also explain the often rather long (up to 50–100 ms) time lag between onset of EMG and rise of IAP.

It is beyond the scope of this article to estimate the relative importance of the abdominal "shock absorber" in relation to the vertebral column with its intervertebral discs. Clearly the importance will depend on a number of factors, some of which can be defined such as the transverse area of the abdominal cavity, the form of the cavity, the actual IAP, the inclination of the body etc. The last factor relates to the location of the mechanical axis. If the mechanical axis goes through the spine, the abdominal cavity will cause little or no loading (Fig. 7 C). If, however, the mechanical axis is ventral to the spine, the abdominal cavity can take up more pressure (Fig. 7 B). The amount of load that can be taken up depends on the stiffness of the walls, which is related to the degree of muscle contraction (see above). The actual load taken up may thus be either high or low when the IAP itself is high (compare Fig. 7 B and C). From this follows that a contraction of the abdominal muscles creating a high IAP (e.g. a Valsalva manoeuvre) does not automatically create an unloading effect on the spine. This may explain why Andersson *et al.* (1976) did not find a decreased disc pressure when Valsalva manoeuvres were induced. In their experiments the course of the mechanical axis is unknown—a further complication may be related to the fact that the pattern of muscle activity inducing a Valsalva (similar to defecation) differs substantially from that occurring e.g. during lifting.

It is noteworthy that an insufficiency in abdominal muscle function has been suggested as one possible factor in the etiology of back pain (e.g. Nachemson 1971). Everything else being equal patients unable to build up adequate IAP would load their spine more

ly in normal movements. Under years of extra loads on the discs and the surrounding tissue this could finally lead to tissue changes resulting in back pain. This possibility is even more likely in view of these experiments, which demonstrate that in normal moments IAP changes occur, which become relatively large under conditions with a physical load on the spine such as when walking down from even a moderate height. It is to be shown, however, that the relevant back patients not only have weak abdominal discs (cf. Nachemson and Lindh 1965) but also tend to build up lower IAP. Another interesting aspect relates to the control of circulation. The increasing IAP will compress the abdominal vessels but should also change the capillary pressure. In addition the phasic variation in IAP will act as a "muscle pump" moving the blood from the legs provided that the venous valves in the legs function. It is noteworthy that with increasing speed the difference between the peak and the trough value increases. This venous pump effect during running should be further explored. Another mechanical effect of the abdominal muscle contraction relates presumably to the need for keeping the centre of mass "in place" during the impact of the body during each contact period.

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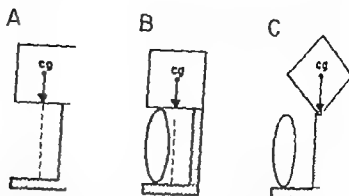


Fig. 7 Schematic representation of how the weight of the upper part of the body may be transferred to the pelvis. A only the vertebral column is assumed to contribute. In B the abdominal cavity takes up a major part of the weight. In C the vertebral column is again solely responsible although an abdominal pressure is built up. *cg.* abbreviation for center of gravity of the upper part of the body

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"Fast" and "slow" skeleto-fusimotor innervation in cat tenuissimus spindles, a study with the glycogen-depletion method

By

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Abstract

JAMIL, L., D. LAN-COUTON, K. MALMÖREN and J. PETIT "Fast" and "slow" skeleto-fusimotor innervation in cat tenuissimus spindles: a study with the glycogen-depletion method. *Acta physiol scand.* 1978. 103. 284-298

The glycogen-depletion method was used to investigate the motor supply to tenuissimus with respect to the presence of fast β axons and to assess the total proportion of both fast and slow β -innervated spindles in this muscle. In the first series of 9 pts., groups of motor axons with conduction velocities higher than 85 m/s were repetitively stimulated so as to produce glycogen depletion in the muscle fibres they innervated. The whole muscle was then quick-frozen, serially cut, stained to demonstrate glycogen and examined for intrafusal glycogen depletion. Zones of glycogen depletion were found in 16 of the 46 examined spindles; they were most frequently located in the longest of the chain intrafusal muscle fibres. Since it is known that there are no purely fusimotor axons to tenuissimus with conduction velocities above 50 m/s, it is concluded that β axons are present among the fastest axons to this muscle. In the second series of 5 experiments, many motor axons as possible with conduction velocities above 60 m/s were stimulated. Zones of glycogen depletion were found in 19 of the 47 examined spindles. They affected chain fibres in about half of the instances and bag fibres in the others. As this latter location is characteristic of slow dynamic β axons, it was concluded that both slow and fast β axons occur regularly in the motor supply to tenuissimus. β -Innervation is present in at least 40% of tenuissimus spindles with almost no convergence of fast and slow β axons onto the same spindle.

Key words: skeleto-fusimotor innervation β axons

Recent evidence indicates that there are two types of skeleto-fusimotor axons, also called β axons. The first and best known type of β axon was originally detected by physiological investigations (Bessou, Emonet-Dénand and Laporte 1963, 1965) and documented shortly after by histological findings (Adal and Barker 1965). Most of these β axons have conduction velocities in the 50-85 m/s range and on stimulation increase the dynamic sensitivity of spindle primary endings (Bessou *et al.* 1965; Emonet-Dénand, Jamil and Laporte 1975; McWilliam 1975). In accordance with these physiological characteristics, slow dynamic

axons were found to innervate intrafusal muscle fibres of the bag type and extrafusal muscle fibres of the slow-oxidative type (Barker *et al.* 1977). Several studies have shown that this "slow" β innervation exists in various muscles of different species (see the review Ennomet-Dénand, Jami and Laporte 1978).

Much less is known about the second type of β axon. Histophysiological evidence for their existence (Barker *et al.* 1977) was first obtained from a study with the glycogen-depletion method of Edström and Kugelberg (1968). This study was carried out on the cat peroneus tertius muscle to investigate whether some motor axons in the 85–110 m/s conduction velocity range give collaterals to muscle spindles. Groups of motor axons with conduction velocities above 85 m/s were repetitively stimulated in order to produce glycogen depletion in the muscle fibres they innervated. The whole muscle was then quick-frozen, sectionally cut, stained to demonstrate glycogen and examined for intrafusal glycogen depletion. Zones of glycogen depletion were found in 27% of the examined spindles, they were almost exclusively located in the longest of the chain intrafusal muscle fibres. As it was further checked that there are no purely fusimotor fast axons to peroneus tertius (see also Dray *et al.* 1972), the intrafusal glycogen depletion provided evidence that some of the motor axons to peroneus tertius in the 85–110 m/s range have a skeleto-fusimotor distribution, i.e., they are β axons. The action of such "fast" β axons on spindle sensory endings is unknown but it can be expected that they are static since they innervate chain fibres.

In the present experiments, similar use was made of the glycogen-depletion method in order to ascertain whether "fast" and "slow" β innervation may coexist in the same muscle. It was first examined whether the cat tenosissimus muscle, already known to receive "slow" β innervation (Barker *et al.* 1971; Ennomet-Dénand *et al.* 1975; McWilliam 1975; Barker *et al.* 1977; Boyd *et al.* 1977) is also supplied by "fast" β axons. As this proved to be the case, an attempt was made to assess the overall incidence of both slow and "fast" skeleto-fusimotor innervation in tenosissimus spindles.

The two types of skeleto-fusimotor axons were termed "fast" and "slow" only on account of their conduction velocities. The fact that each type of β axon innervates a different type of extrafusal muscle fibre further suggests that they serve different functions, which appears to justify their distinction on the basis of conduction velocity. But it should be kept in mind that the limit of 85 m/s between "fast" and "slow" β axons was set empirically so that some overlap is to be expected. In the present study the term "slow" β axon will be used as a synonym of dynamic β axon supplying bag intrafusal muscle fibres, whereas the term "fast" β axon will designate a β axon of unknown function supplying chain intrafusal muscle fibres.

Methods

Two series of 5 experiments each were carried out on adult cats anesthetized with pentobarbital (Nembutal, Abbott Laboratories, 40 mg/kg I.P.). The first series was aimed at the detection of fast β axons innervating the tenosissimus and in each experiment of this series as many motor axons as possible with conduction velocities above 85 m/s were stimulated simultaneously (see Fig. 1 and Table I). The aim of the second series of experiments was to determine the total number of tenosissimus spindles innervated by β axons, whether slow or fast. This implied the simultaneous stimulation of all motor axons to tenosissimus with the sole exception of γ axons, in practice, as many motor axons as possible with conduction velocities above 30 m/s were selected for stimulation in each experiment of this series (see Fig. 4 and Table II).

"Fast" and "slow" skeleto-fusimotor innervation in cat tenuissimus spindles, a study with the glycogen-depletion method

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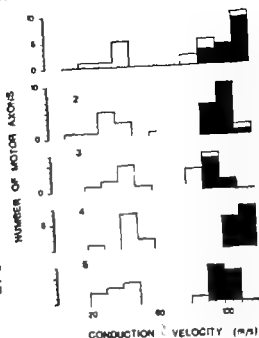
Abstract

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The glycogen-depletion method was used to investigate the motor supply to tenuissimus spindles with respect to the presence of fast β axons and to assess the total proportion of both fast and slow β -innervated spindles in this muscle. In the first series of 5 experiments, groups of motor axons with conduction velocities higher than 35 m/s were repetitively stimulated so as to produce glycogen depletion in the muscle fibres they innervated. The whole muscle was then quick-frozen, serially cut, stained to demonstrate glycogen and examined for intrafusal glycogen depletion. Zones of glycogen depletion were found in 16 of the 46 examined spindles; they were most frequently located in the longest of the chain intrafusal muscle fibres. Since it is known that there are no purely fusimotor axons to tenuissimus with conduction velocities above 50 m/s, it was concluded that β axons are present among the fastest axons to this muscle. In a second series of 5 experiments, many motor axons as possible with conduction velocities above 60 m/s were stimulated. Zones of glycogen depletion were found in 19 of the 47 examined spindles. They affected chain fibres in about half of the instances and bag fibres in the others. As this latter location is characteristic of slow dynamic β axons, it was concluded that both slow and fast β axons occur regularly in the motor supply to tenuissimus. β -innervation is present in at least 40% of the tenuissimus spindles with almost no convergence of fast and slow β axons onto the same spindle.

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Recent evidence indicates that there are two types of skeleto-fusimotor axons, also called β axons. The first and best-known type of β axon was originally detected by physiological investigations (Bessou, Emonet-Dénand and Laporte 1963, 1965) and documented shortly after by histological findings (Adair and Barker 1965). Most of these β axons have conduction velocities in the 50-85 m/s range and on stimulation increase the dynamic sensitivity of spindle primary endings (Bessou *et al.* 1965; Emonet-Dénand, Jami and Laporte 1975; McWilliam 1975). In accordance with these physiological characteristics, slow dynamic



1. Conduction velocities of the motor axons examined in the 5 experiments of the first series. Stimulated axons are represented by filled columns.

examined spindles and the number of spindles in which glycogen depletion was found. It is well-known that the motor supply to tenuissimus does not comprise purely α -motor axons with conduction velocities above 50 m/s (Kuffler, Hunt and Quilliam '71) and since there was no γ axon among the stimulated axons, intrafusal glycogen depletion can in every case be considered evidence of fast β innervation.

Depleted intrafusal muscle fibres were observed in 16 spindles out of 46 examined (34%). Most of them were chain fibres as previously reported for the peroneus tertius muscle (Barber *et al.* 1977). A schematic reconstruction of one of the depleted spindles is shown in Fig. 2. The longest chain fibre was depleted in both poles, mainly in its intracapsular portions. This chain fibre extends 1 mm beyond the end of the capsule at one pole and even further at the other: it is therefore a "long chain" according to the criterion of Barber *et al.* (1976).

Table 1. Number of tenuissimus spindles supplied by skeleto-fasciomotor axons with conduction velocity higher than 85 m/s.

Exp. No.	Number of stimulated motor axons (range of conduction velocities)	Number of examined spindles	Number of spindles with zones of glycogen depletion
1	18 (90-112 m/s)	13	4
2	12 (85-112 m/s)	10	2
3	9 (85-107 m/s)	7	2
4	16 (90-113 m/s)	6	3
5	13 (90-105 m/s)	10	3
Total		46	16

The essentials of the experimental methods were similar to those fully described by Barker *et al.* (1971). All the hind limb muscles were denervated except the tenoissimus whose nerve, as dissected here, was 10–15 mm and mounted on a recording electrode. The second electrode was placed in contact with the preparation at some distance from the tenoissimus. This made possible the recording through the cut channel of the nerve and muscle action potentials elicited by the stimulation of motor axons in ventral roots.

The L7 and S1 ventral roots, which supply the tenoissimus, were split into small filaments and conduction velocities of all motor axons to this muscle (γ as well as α), were measured. Only those filaments containing axons with conduction velocities above either 85 m/s (in the first series) or 60 m/s (in the second series) were kept for stimulation. All the filaments with slower axons were severed as close as possible to the dura. In order to facilitate the elimination of filaments containing γ axons, a severe lignocaine block of nerve conduction as described by Barker *et al.* (1977) was used during the preparation of ventral root filaments in 3 expts. of the first series and 2 expts. of the second series.

The number of axons identified in each expt. ranged from 9 to 21 (average 16) for the α axons and from 7 to 14 (average 9) for the γ axons.

After completion of ventral root exploration all the prepared filaments were mounted on a multi-wire stimulating electrode so as to be simultaneously excited. The nerve block was removed by washing the preparation with Ringer's solution and within an hour the conduction appeared fully restored in all nerve axons.

The regime of stimulation used to elicit glycogen depletion was similar to that used by Barker *et al.* (1977 see also Barker *et al.* 1977). Stimulation at 30 Hz was applied for one minute while the blood supply to the muscle was reduced by pulling on the femoral iliac artery with a rubber band. Stimulation was stopped for the following minute while a normal blood flow was allowed through the limb. The sequence was repeated 16 times without interruption and the muscle was excited during the total period of stimulation and blood occlusion.

The nerve action potentials were constantly monitored during stimulation in order to make certain that no stray γ axon was excited together with the selected α axons, as may occur when γ axons recover from damage suffered during ventral root splitting. The absence of γ stimulation was finally checked by recording action potentials of the stimulated axons from the nerve stump after removal of the muscle.

A few seconds after excision, the muscle was quenched in isopentane cooled to -160°C . It was then stored at -30°C . The following day it was divided into portions that were cut frozen in serial transverse sections 25 μ thick. The sections were stained for glycogen using the PAS reaction and examined for glycogen depletion. The total length of muscle sectioned in each experiment (38 to 61 mm) represented from one quarter to one half of the whole muscle.

Serial reconstructions of spindles showing glycogen depletion were drawn in order to map (1) depleted zones in the intrafusal muscle fibres. The 3 types of fibre (bag, bag₂ and chain fibres) were identified on the basis of length, diameter, equatorial association and glycogen content, according to the criteria established by Banks, Harker and Stacey (1977). In a few instances, intrafusal muscle fibres which appeared paler than normal were observed suggesting that glycogen might have been incompletely depleted in them. Such zones occurring in bag₂ fibres will be reported but in the final counts only those fibres showing zones of complete depletion were considered as being β -innervated.

Results

Spindles innervated by "fast" β axons in tenoissimus

The conduction velocities of the motor axons to tenoissimus identified in each experiment of the first series are shown in Fig. 1. All the stimulated axons in this series had conduction velocities above 85 m/s (expts. 2 and 3) or 90 m/s (expts. 1, 4 and 5). Their number ranged from 9 to 18 which represented, on average, 93% of the axons with conduction velocities superior to 90 m/s present in the muscle nerve.

In each expt. glycogen depletion was observed in several spindles after prolonged stimulation of a group of very fast conducting motor axons. For each of the 5 expts. Table I gives the number of axons stimulated and their conduction velocities. The number

	Data	Pole	Proximal	Po
	In	Ex	Ch	
1	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
2	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
3	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
4	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
5	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	
	○ ○	○ ○	○ ○	

3 Summary of the glycogen depletion elicited in 16 motor spindles by stimulating groups of motor axons conducting velocities higher than 85 m/s. A few spindles were depleted. The numbers on the left are the different experiments. Medium-diameter circles (light shading) represent non-depleted poles of bag, ex, large circles (shading) those of bag, ex, and small filled circles those of chain fibres. In a spindle chain fibres are arranged in order of decreasing length. Fibres that contained zones of glycogen depletion are represented by open circles. Open circles with a question mark represent bag, fibres that showed an incomplete partial depletion (see text).

tested two fibres which were the two longest chain fibres in two cases and the longest and with longest chain fibres in the other. It was also the longest chain fibre that was involved in the 3 poles where bag fibres were depleted in addition to chain fibres. In summary however a spindle pole showed chain fibre depletion, the longest chain fibre was affected (see also Harker *et al.* 1977).

Of the 16 depleted spindles, only two were depleted in both poles and all the affected fibres were depleted in one zone only. In total 9 spindles had a single zone of depletion and 7 had two zones. The mean length of the depleted zones in the long chain or in the longest chain was 533 μ m (for 16 zones ranging from 225 to 1400 μ m). The lengths of the depleted zones located on other chain fibres were 200, 450 and 300 μ m respectively. For the 3 zones located on bag, fibres they were 325, 450 and 500 μ m and for the 2 zones located on bag, fibres 125 and 1500 μ m respectively.

Measurement of the total number of β -innervated tenubusmus spindles

For the second series of experiments, cats could not be chosen randomly as was done for the first series, because axons in the 60–80 m/s conduction velocity range are often absent from the motor supply to cat tenubusmus. In such cases the lack of slow-conducting axons could be inferred first from the shape of the tenubusmus nerve action potential recorded on stimulation of L7 and S1 ventral roots prior to any splitting. It was confirmed afterwards during ventral root examination, as can be seen in Fig. 1 where histograms 1, 4 and 5 show gaps in this range. In fact, 7 out of 14 examined cats showed similar gaps. Since the principle of the experiments implied the stimulation of as many as possible motor axons with conduction velocities covering the whole range of 60 to 110 m/s, it appeared

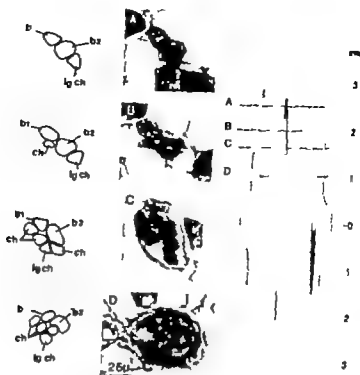


Fig. 2. Glycogen depletion elicited in a tenuisimus spindle by the simultaneous stimulation of 11 axes with conduction velocities higher than 85 m/s (expt. 2). *Left*: Microphotographs of spindle transverse sections stained to demonstrate glycogen (PAS method). In A the level of sectioning is near the top of the spindle pole; the bag₁ (b1) and bag₂ (b2) fibres retain medium levels of glycogen, while that of the long chain (lg ch) fibre is low. In B the level of sectioning is still extracapsular: the long chain is depleted, whereas the other fibres show normal levels of glycogen, i.e. medium for bag₁, medium/high for bag₂ and high for chain (ch) fibre. In C the level of sectioning is intracapsular: the long chain is depleted, whereas bag fibres and the 3 other chain fibres show normal levels of glycogen. In D the level of sectioning is intracapsular and the long chain has recovered a normal content of glycogen. *Right*: Schematic reconstruction of the spindle showing the pattern of glycogen depletion. The light-shaded, coarse-shaded and blank intracapsular fibres are the bag₁, bag₂ and chain fibres, respectively. The blank stretches on the long chain indicate the zones of glycogen depletion. The dashed lines show the levels of transverse sectioning. The scale applies only to the length of the intrafusar muscle fibres.

The patterns of depletion observed in the 16 spindles are summarized in Fig. 3. These spindles contained a total of 32 bag fibres (one of each type per spindle) and 73 chain fibres (mean chain fibre complement 4.5 per spindle). In 11 spindles, only chain fibres were depleted. In 2 other spindles the bag fibre was depleted in addition to chain fibres and in another spindle the bag and a chain fibre were affected together. In the remaining 3 spindles (both from the same experiment) the bag was the only fibre showing a zone of complete depletion but in the portion of the bag₂ fibre that lay side-by-side with the depleted zone the glycogen content dropped to an extremely low level suggesting that some activation of the bag fibre also might have occurred. In total, zones of complete depletion were found in 18 spindle poles, with chain fibres being involved in 16 poles, bag₁ fibres in 3 and bag₂ fibres in 2.

In the 16 poles with chain fibre depletion the distribution of depleted zones was as follows: in 13 poles a single chain was depleted which was invariably either a long chain (6 poles had such a fibre) or the longest chain fibre present. In 3 poles, depletion

	Dist	Pole	Pr	mal	Pole
	to	to	ch		
6	{			○ ⊗ ○ ○ ○ ○	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○	
		○ ⊗		○ ⊗ ○ ○	
7	{	○ ⊗		○ ⊗ ○ ○ ○ ○	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○	
		○ ⊗ ○ ○ ○ ○ ○ ○		○ ⊗ ○ ○ ○ ○	
8	{	○ ⊗ ○ ○		○ ⊗ ○ ○ ○	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○	
9	{	○ ⊗ ○ ○ ○		○ ⊗ ○ ○ ○ ○ ○ ○	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○ ○ ○ ○ ○	
		○ ⊗		○ ⊗ ○ ○ ○ ○ ○	
		○ ⊗ ○ ○		○ ⊗	
10	{	○ ⊗ ○ ○		○ ⊗	
		○ ⊗ ○ ○		○ ⊗ ○ ○ ○	
		○ ⊗		○ ⊗	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○	
		○ ⊗ ○ ○ ○ ○		○ ⊗ ○ ○ ○ ○	

1. Summary of the glycogen depletion elicited in 19 muscle spindles by stimulatory groups of motor axons at conduction velocities higher than 60 m/s. The conventions as for Fig. 1. In expt. 6 the distal 10 of 104 spindles were lost.

spindles. For the reason already mentioned—absence of purely fusimotor axons in the selection velocity range—the depleted spindles may be considered as being β -innervated. In 17 of the 19 spindles both poles could be examined, but in 2 spindles, only one pole was available because of damage caused by division of the frozen muscle into small portions prior to sectioning. The patterns of depletion observed in these 17 whole spindles and 11 half spindles are summarized in Fig. 5. The 17 whole spindles contained a total of 34 bag fibres (one of each type per spindle) and 77 chain fibres (mean chain fibre complement 4.5 per spindle). The half spindles contained a total of 4 bag fibre poles and 7 chain fibre poles. In 9 whole spindles and in one half spindle, depletion occurred only in chain fibres. In 7 other whole spindles the bag was the only depleted fibre. In each of the remaining whole spindle and half spindle the bag, and a chain fibre were affected together. It should also be mentioned that in 3 spindles the bag, fibre showed a zone of partial depletion, zone of low glycogen content, which had the same length and the same location as the totally depleted zone of the neighbouring bag, fibre (in two spindles) or chain fibre (in the third spindle). In total, zones of complete depletion were found in 21 spindle poles with chain fibres being involved in 11 poles and bag, fibres in 10.

In keeping with previous observations (see the first series of expts. See also Harker *et al.* 1977) however spindle pole showed chain fibre depletion it affected the longest of chain fibres, which actually was "long chain" in 5 cases. In 2 spindle poles, depletion affected the second longest chain fibre in addition to the longest one.

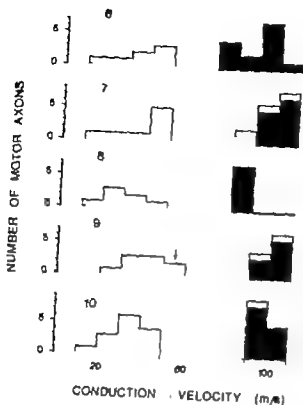


Fig. 4. Conduction velocities of the motor axons to tenuissimus in the 5 expts. of the second series. The stimulated axons are represented by filled rectangles. In histogram 9 the arrow points to 2 non-stimulated axons with conduction velocities between 60 and 80 m/s.

necessary to select those cats whose tenuissimus was supplied also by some motor axons in the lower end of the range (Fig. 4). The selection probably explains the difference between the distributions of conduction velocities in the two series of experiments. The average proportion of axons having a velocity higher than 90 m/s was 85% and 65% in the first and second series respectively (compare Fig. 4 with Fig. 1). Whether this sampling bias interfered with the results will be considered in the discussion.

Table II gives the number and conduction velocities of the stimulated axons, the number of examined spindles and the number of spindles in which glycogen depletion was found for each of the 5 expts. in this second series. The number of stimulated axons represented on average 92% of the axons with conduction velocities superior to 60 m/s present in the muscle nerve. Intrafusal depletion was observed in 19 (40%) of the 47 examined

Table II. Number of tenuissimus spindles supplied by skeleto-fusimotor axons with conduction velocities higher than 60 m/s.

Exp. No.	Number of stimulated motor axons (range of conduction velocities)	Number of examined spindles	Number of spindles with zones of glycogen depletion
6	14 (70-108 m/s)	9	4
7	15 (60-109 m/s)	7	3
8	9 (60-111 m/s)	8	2
9	11 (70-107 m/s)	11	4
10	17 (60-109 m/s)	12	6
Total		47	19

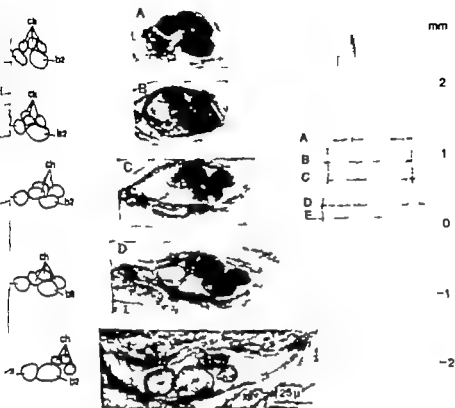


Fig. 6B

Spindle *ch* bag, fibre depleted in both poles. *Left* The microphotographs of spindle transverse sections show that the bag, fibre has normal glycogen level (medulla) in A, both deplete to low in B. In bag, fibre depleted in C and again low in D. The section in E is very near the equator so that B the bag fibres appear paler than at polar levels. *Right* schematic reconstruction of the spindle showing the areas of glycogen depletion in both poles of bag, fibre. Dashed lines indicate the levels of transverse sections.

in the longest chain fibre was 480 μm (for 12 zones ranging from 150 to 950 μm). The length of the two zones located on the second longest chain fibres were 200 and 375 μm respectively. The mean length of the depleted zones in the bag, fibre was 431 μm (for 8 zones ranging from 225 to 725 μm).

The main difference between the results of the two series of expts. lies in the intrafusar distribution of the zones of glycogen depletion. Table III shows that in all the expts. of the second series except one (n° 9), spindles with only chain depletion and spindles with only bag, depletion are found in the same muscle. Schematic reconstructions of two such spindles are shown in Fig. 6. In spindle A the pattern of depletion observed in the "long chain" is quite similar to the patterns observed in the first series of expts. (see also Barker *et al* 1977), while the bag, depletion in spindle B compares with the patterns of depletion produced by stimulation of slow dynamic β axons (see Fig. 2 in Barker *et al* 1977). It is

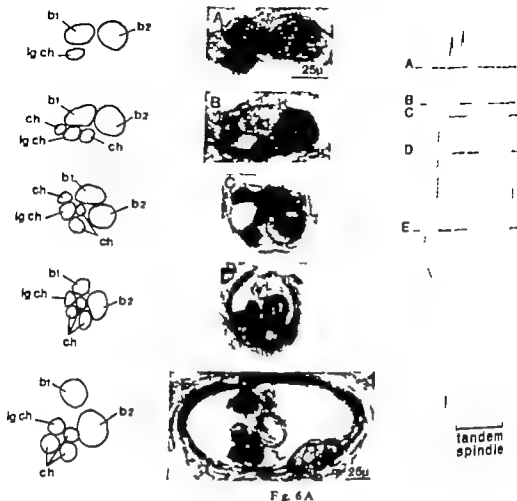
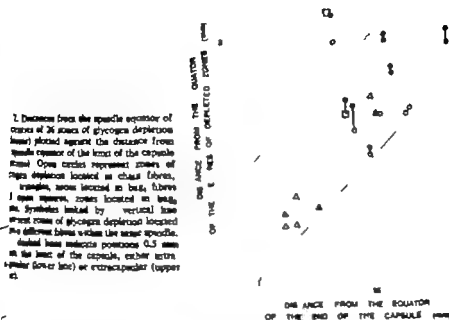


Fig. 6A

Fig. 6. Zones of glycogen depletion elicited in two different spindles from the same tenrisomes and by the simultaneous stimulation of 17 motor axons with conduction velocities higher than 60 m/s (eq. 10). Same conventions as for Fig. 2.

A. Spindle with a long chain depleted in both poles. *Left* Microphotographs of spindle transverse sections stained to demonstrate glycogen (PAS method). The scale in A is also valid for B, C and D. In A the level of sectioning is near the tip of the spindle pole the level of glycogen is medium for the bag fibres and high for the long chain fibre. In B the level of sectioning is intracapsular the level of glycogen in the long chain fibre has dropped to medium/low the bag fibre is medium the bag₂ fibre is medium/high and the two other chain fibres are high. In C the level of sectioning is near the limit of the capsule the long chain is depleted whereas the 3 other chain fibres are normally high. Note that the level of glycogen are now medium/high and medium in bag and bag₂ fibres respectively. In D the level of sectioning is intracapsular the long chain fibre is still depleted and the other chain fibres are normally high. The level of glycogen in bag₁ and bag₂ fibres have reversed to medium/low and medium/high respectively. Note the general reduction of intrafusal muscle fibre diameters. In E the level of sectioning is juxta-equatorial, the long chain has recovered a medium/high level of glycogen. Note the association of bag₂ and chain fibres. *Right* Schematic reconstruction of the spindle showing the zones of glycogen depletion in both poles of the long chain fibre. Dashed lines indicate the levels of transverse section. The bag₂ and 2 chain fibres passed to another spindle linked in tandem to this spindle.

Of the 17 whole spindles only 2 were depleted in both poles, with the bag fibre being involved in one spindle and the "long chain" in the other. As in the first series, all the affected fibres were depleted in one zone only. 12 of the whole spindles had a single zone of depletion and 7 had two zones. The mean length of the depleted zone was 1.5 mm. long chain



spindles in both chain fibres were depleted. Altogether the positions of the depleted spindles may be explained by assuming that in most instances there was either a single fast β axon or a single fast β axon plus a single slow β axon among the stimulated axons.

Discussion

The present experiments show that in tennisimus muscle fast skeleto-fusimotor axons with conduction velocities above 85 m/s participate in the innervation of spindles and specifically of the longest intrafusal chain muscle fibres. The intrafusal patterns of glycogen depletion produced by fast β axons in tennisimus were roughly similar to those found in peroneus tertius by Barker *et al.* (1977). Fast β axons occur regularly in the motor supply to tennisimus since they were found in all expts. of both series. Since slow dynamic β axons have been previously demonstrated in tennisimus (Barker *et al.* 1971; Emonet-Dibard *et al.* 1975; M. Williams 1975; Barker *et al.* 1977; Boyd *et al.* 1977) the first series of expts. suggests that both types of skeleto-fusimotor innervation may be present in this muscle. The actual coexistence of fast and slow β axons in the same muscle was demonstrated in the expts. of the second series.

Spindles showing the typical feature of slow β innervation, namely bag fibre depletion (Barker *et al.* 1977), were found in all expts. of the second series except one (n. 9). However this exception does not prove that slow β axons to tennisimus are inconstant, because histogram 9 in Fig. 4 shows that in this expt. 2 motor axons with conduction velocities

TABLE III Intrafusal distribution of zones of glycogen depletion.

Exp No	Number of spindles in which depletion was located on			
	chain fibres only	chain fibres + bag fibre	chain fibres + bag fibre	bag fibre only
1	2			2
2	2			
3	2			
4	2	1		
5	3	1	1	
Total	11	2	1	2
6	1		2	1
7	1			2
8	1			1
9	4			
10	3			3
Total	10		2	7

thus very likely that in the present sample the spindles showing bag fibre depletion are innervated by slow β axons.

It is generally accepted—although not proven—that each zone of glycogen depletion found in intrafusal muscle fibre lies around the motor ending of the stimulated axon. The positions of the depleted zones might thus indicate the sites of termination of β axons. In Fig. 7 the distances from the spindle equator of the centres of the zones of glycogen depletion are plotted against the distance from the equator of the limit of the capsule to each spindle pole. Results from both series of experiments have been pooled showing that nearly all the centres of depleted zones were located within half millimeter from the end of the capsule either inside or outside the capsule sleeve. Similar locations of the zones of glycogen depletion elicited by stimulation of fast β axons in peroneus tertius spindles have been reported by Harker *et al.* (1977).

The number of β axons that were stimulated in either series of expts. is difficult to estimate for the following reasons. The only criterion for selecting the axons to be stimulated was their conduction velocity but it was impossible to ascertain that all the axons in the chosen range of conduction velocities were actually identified and prepared for stimulation. Table I and II compared to Fig. 1 and 4 show that there was no correlation between the proportion of stimulated axons and the proportion of depleted spindles. Moreover it is known that a single slow dynamic β axon can supply 4 spindles (Barker *et al.* 1977), but the average number of spindles innervated by a single fast β axon is unknown. However the number of β axons stimulated in the present expts. may be indirectly estimated from the position of depleted spindles in the examined portions of muscles. Fig. 8 shows that in experiment 5 for instance, the five depleted spindles were found within a portion of muscle inferior to 25 mm in length. Such close grouping of depleted spindles is rather suggestive of their being innervated by a single fast β axon. In expts. 7 and 10, spindles with bag fibre depletion also appear in close grouping and at some distance from the

However since some spindles with bag₁ fibre depletion were found in expts. 1 and 5 (Table III) the possibility should not be overlooked that some dynamic β axons may have been stimulated. Histograms 1 and 5 in Fig. 1 show wide gaps in motor axon action velocities between 50 and 80 m/s which makes not unlikely the presence of some β axons in the higher range of conduction velocity in these two experiments. Fast-conducting dynamic β axons to tenosSESUS have already been observed since in a sample studied by McWilliam (1975) the conduction velocities ranged from 63 to 75 m/s.

The possibility of dynamic β axon activation in some experiments of the first series is also account for the fact that the intrafusal distribution of fast β axons appears less active in tenosSESUS than in peroneus tertius. In the latter muscle glycogen depletion was noted in chain fibres in 90% of the depleted spindle poles (Harker *et al.* 1977) as against 5% in the present sample. In any case, the specific innervation of the longest chain fibre seems an intriguing feature of fast β axons. Their intrafusal distribution suggests that they exert static actions on spindle sensory endings but their selectivity for chain fibres contrasts with the non-selective distribution of static γ axons (see the discussion in Harker *et al.* 1977).

A further question is whether the observed patterns of glycogen depletion fully account for the intrafusal distribution of the stimulated axons. In their γ -depletion study Barker *et al.* (1976b) noted that complete depletion of chain fibres sometimes appeared difficult to achieve. This might also apply to bag₁ fibres which in the present sample showed more instances of partial depletion (expts. 1, 6, 9 and 10) than of complete depletion (expts. 2 and 5). Dynamic β axons only exceptionally depleted bag₁ fibres (Barker *et al.* 1977) and fast β axons to peroneus tertius were never observed to do so (Harker *et al.* 1977). But evidence has not been provided so far that bag₁ fibres might be deprived of β innervation. On the contrary the p₁ type of intrafusal motor end plates, which are considered as the terminations of β axons (Barker, Stacey and Adal 1970), have been found in almost equal proportions on both types of intrafusal bag fibres in various hind-limb muscles of the rabbit (Barker and Stacey 1970). Moreover the stimulation of static γ axons quite readily depleted zones of glycogen depletion in bag₁ fibres (Barker *et al.* 1976). Since fast β axons are probably static, it is surprising that when activated they should prove unable to deplete bag₁ fibres.

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Fig. 8. Positions of depleted spindles in the extensor posterior tenotomus muscles. The vertical line indicates the depletion zone for block represents the length of a portion of muscle examined. Depleted spindles are represented by horizontal bars, those in which zones of glycogen depletion were located on the bag, fibre or marked by open triangles.

between 50 and 60 m/s were not stimulated (see arrow) and the possibility cannot be excluded that these axons were skeleto-fusimotor. It is therefore likely that slow β axons occur in tenuissimus as regularly as fast ones. In fact, if expt 9 is removed from the results of the second series the total proportion of depleted spindles in the sample (15 of 36 spindles see Table III) comes out as 42% with approximately equal numbers of fast and slow β -innervated spindles (*i.e.* 6 spindles with only chain depletion and 7 with only bag depletion). The 2 spindles in which zones of glycogen depletion were located in both bag and chain fibres might be allotted to either type of β innervation since it is difficult to decide whether they represent occasional variations from regular patterns—such variations were observed in both of the glycogen-depletion studies of slow and fast β innervation (Barker *et al.* 1977; Harker *et al.* 1977)—or whether they are instances of slow and fast β axons converging onto the same spindle. The small number of spindles showing this pattern of depletion indicates that for unknown reasons, such convergence is rare. Allowing for this uncertain convergence, the proportion of slow β -innervated spindles in the second series of expts (n = 9 excluded) is at most 25% of the total sample and that of the fast β -innervated spindles is at most 22%.

However, this proportion of tenuissimus spindles supplied by slow β axons (25%) is probably still an underestimate due to the fact that the lower limit of conduction velocity for the stimulated axons was set at 60 m/s in these expts. It is known that the slowest dynamic β axons can be found in the γ range of conduction velocities (Bessou *et al.* 1965; Emonet-Dénand *et al.* 1975) but if present in the studied muscles, such dynamic β axons would have been excluded from the group of stimulated axons. The probability of very slow β axons occurring in our sample is not negligible because of the sampling bias whose effect appeared to shift down by about 10 m/s the whole range of conduction velocities of motor axons to tenuissimus (compare Fig. 4 with Fig. 1). The assumption that the proportion of slow β -innervated tenuissimus spindles might be superior to 25% is further supported by the findings of McWilliam (1975) who reported a proportion of 30% and considered it as a slight underestimate.

The assessed proportion of tenuissimus spindles innervated by fast β axons in the second series of expts (22%) does not compare with the 34% depleted spindles found in the first

Bronchial and cardiovascular actions of prostaglandin endoperoxides and an endoperoxide analogue

By

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Abstract

HEDQVIST P. K., STRANDBERG K. and HANBERG M. *Bronchial and cardiovascular actions of prostaglandin endoperoxides and an endoperoxide analogue* Acta physiol. scand. 1978; 103: 299-307.

Effects of PGG_2 , PGH_2 and the endoperoxide analogue, EPA, on bronchial and vascular smooth muscle are studied *in vivo* and *in vitro*. 1. The cat PGG_2 , PGH_2 and particularly EPA proved potent stimulants of airway resistance, and they were all significantly more active than $\text{PGF}_{2\alpha}$. They also increased pulmonary vascular resistance but EPA alone as more active than $\text{PGF}_{2\alpha}$. 1 $\mu\text{g/kg}$ *ip* EPA was 90-190 times more active than $\text{PGF}_{2\alpha}$ in increasing tracheal inflation pressure. Human bronchi contracted in response to PGG_2 , PGH_2 and EPA. PGG_2 and PGH_2 were equiactive with $\text{PGF}_{2\alpha}$ and EPA was more than 100 times as potent. PGG_2 and PGH_2 lowered systemic arterial blood pressure and increased heart rate. They were as active or more active than PGE_2 . EPA, on the other hand, resembled $\text{PGF}_{2\alpha}$ in producing biphasic changes of blood pressure and heart rate or decreases of blood pressure and bradycardias in paws *ip* and cat. The results obtained in this study indicate that PGG_2 , PGH_2 and EPA are potent stimulants of bronchial and pulmonary vascular smooth muscle. The data are also consistent with the view that significant proportion of effects resulting from prostaglandin generation in the lung is due to prostaglandin endoperoxides rather than primary prostaglandins.

The prostaglandin (PG) endoperoxides, PGG_2 and PGH_2 , intermediates in the biosynthesis of PGE_2 , $\text{PGF}_{2\alpha}$ and thromboxane A_2 (TxA_2) (Hamberg and Samuelsson 1973; Nugteren and Hazelhof 1973; Hamberg *et al.* 1975 b), have been shown to be potent constrictors of airway and vascular smooth muscles (Hamberg *et al.* 1975). However, due to the rapid conversion of the endoperoxides into their endproducts it was difficult to assess the quantitative or even qualitative actions of the endoperoxides *per se*. In this communication we report the actions of PGG_2 , PGH_2 the synthetic stable endoperoxide analogue, 15(S)-hydroxy-9 α , 11 α -(epoxymethano)prosta-5,13-dienoic acid (EPA), as well as some degradation products of the endoperoxides on the respiratory and cardiovascular systems in cats and guinea pigs, and on isolated human bronchi. A preliminary account of part of the results has been presented elsewhere (Hedqvist *et al.* 1974).

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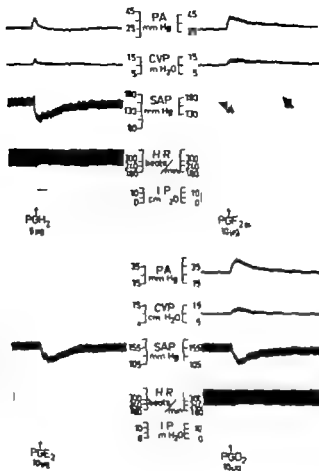


Fig. 1. Effects of intravenously administered PGE_2 , PGE_3 , PGE_2 +EPA and PGE_2 + PGE_3 on pulmonary arterial pressure (PA), central venous pressure (CVP), systemic arterial pressure (SAP), heart rate (HR), and tracheal ventilation pressure (IP) cat.

whereas PGE_2 and PGE_3 and PGE_2 and PGE_3 equally active on I. and Ia. administration (Table III). PGE_2 affected the systemic arterial blood pressure in a different manner. In most cases it caused a biphasic response, either an initial drop followed by a moderate and more long-lasting increase (Fig. 1), or the reverse pattern. However in some expts. the effect of

Table III. Active activity of PGE_2 , PGE_3 , PGE_2 +EPA and PGE_2 + PGE_3 on pulmonary arterial pressure (P_{PA}) and tracheal ventilation pressure (P_{TA}) in cats. Mean values \pm S.E.

	PGE_2	PGE_3	PGE_2	EPA	PGE_2
P_{PA}	9.54 ± 0.16 6 P 0.01	1.21 ± 0.40 5	1.08 ± 0.27 9 P 0.01	33.9 ± 8.9 15 P 0.01	1.0
P_{TA}	1.33 ± 0.36 12	2.33 ± 0.29 12 P 0.01	4.43 ± 0.54 17 P < 0.01	44.9 ± 10.9 25 P < 0.01	1.0

Methods

Cats of either sex (2.5–4.0 kg) were anesthetized with sodium pentobarbitone (30 mg/kg) in supplementary doses were administered i.v. as required to maintain anesthesia. The trachea was cannula and artificial respiration was administered with a Harvard small animal respirator. Spontaneous breath was precluded with gallamine triethiodide (Flaxedil[®]) (1 mg/kg every 30 min). Changes in tracheal insufflation pressure were recorded by means of a Statham pressure transducer (P23 BC) connected to its side arm of the airway circuit (1 ltr), and a Grass Model 5D polygraph. The main pulmonary artery was approached through a leftward thoracotomy and a thin indwelling polyethylene catheter was placed in the artery. Polyethylene catheters were also inserted in the right femoral vein and artery and advanced to the abdominal part of v. cava and the aorta, respectively. Blood pressures were recorded with Statham pressure transducers (P23 BC for central venous pressure and P23 AC for pulmonary artery and systemic pressure) and the Grass polygraph. The heart rate was measured by the use of an ordinate meter triggered by the arterial pulse wave. Test agents were injected into the right jugular vein or the aortic arch.

Male albino guinea pigs (500–700 g) were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and were prepared for registration of tracheal insufflation pressure as previously described in detail (Strandberg and Hedqvist 1973). Briefly the animals were ventilated with Palmer constant volume respirator (8 strokes/min, 4–6 ml/kg b.w.) and changes in insufflation pressure were measured with a Statham pressure transducer (P23 BC) connected to the tracheal cannula. Systemic arterial blood pressure and heart rate were monitored from the right carotid artery using a Statham pressure transducer (P23 AC). All parameters were recorded on a Grass 5D polygraph. At the beginning of the experiment heparin (300 IU/kg) was given into the right jugular vein. This route was also used for administration of test agents and metabolic supplements.

Human bronchi were free-dissected from macroscopically healthy pulmonary tissue obtained at connect on with surgery for pulmonary tumours. Helical strips of bronchi were either kept in 5 ml organ baths containing Tyrode's solution or superfused at a rate of 1.5 ml/min with Tyrode (conc. in mM: NaCl 136.7, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaH₂PO₄ 0.4, NaHCO₃ 11.9, glucose 5.5), equilibrated at 37°C and gassed with 5% CO₂ in O₂. Isometric recordings of the smooth muscle activity were obtained using a force displacement transducer (Grass FT 03) with a load of 0.5 g and a Grass 5D polygraph.

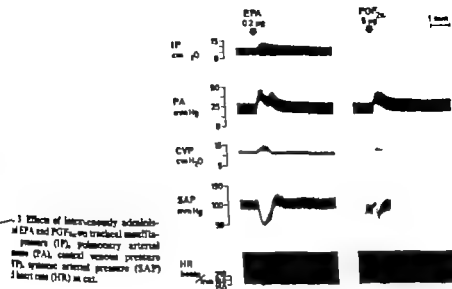
PGG₂ and PGH₂ prepared according to Hamberg *et al.* (1974), were kept in acetone (1 µg/µl) at 20°C and diluted with 0.9% NaCl immediately before use. The final concentration of acetone did not influence any of the studied parameters. PGE₂, PGE₁, PGF_{2α} as well as EPA (Boody 1975) were generously supplied by Dr J. Pike, Upjohn Co., Kalamazoo, Michigan.

Statistical analysis was performed according to Student's *t*-test for paired and unpaired data.

Results

Cat experiments

Fig. 1 illustrates some cardiovascular and bronchial effects of the endoperoxide PGG₂ and some of its possible degradation products. Upon injection into the right jugular vein PGG₂, PGD₂ and PGF_{2α} (5–10 µg) regularly increased pulmonary arterial pressure, central venous pressure and tracheal insufflation pressure, whereas PGE₂ in the same concentrations only slightly and inconsistently affected these parameters. Comparison of relative activities of endoperoxides and degradation products on pulmonary arterial pressure revealed that PGG₂, PGG₂ and PGF_{2α} were of the same potency and PGD₂ less active (Table I). On the other hand the rank order of potency on tracheal insufflation pressure was PGG₂ = PGG₂ > PGF_{2α} = PGD₂ (Table I). Upon i.v. administration PGD₂, PGE₂, PGE₁ and PGG₂ all reduced systemic arterial blood pressure and increased the heart rate (Fig. 1 Table II). PGD₂ was less and PGG₂ significantly more effective than PGE₂. On the other hand PGE₂ was as active a vasodepressor as PGG₂ when the two compounds were given i.a. Comparison of i.v. and i.a. responses revealed that PGG₂ was more active



single EPA was used. EPA doses of the order of 0.1–0.5 μg sufficed to markedly increase tracheal insufflation pressure and pulmonary arterial pressure. In the latter case a two peak response was often observed, suggesting recirculation of intact EPA (Fig. 3). Relative to PGF_{2α} EPA was 30–40 times more potent on tracheal insufflation pressure and pulmonary arterial pressure (Table I). As with PGF_{2α} EPA produced mixed effects on systemic arterial pressure. The most prevalent response was an initial drop followed by a moderate increase, although pure depressor or pressor responses were also seen. The effect of EPA on the heart rate is consistently that of a slight decrease.

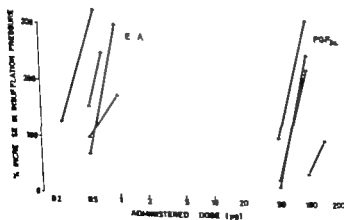


Fig. 4 Dose-response relations for intravenously administered PGF_{2α} and EPA on tracheal insufflation pressure in 4 guinea pigs.

TABLE II Relative depressor activity of intravenously administered PGD_2 , PGG_2 , PGI_2 , and PGE_2 on systemic arterial pressure in cats. Mean values \pm S.E.

PGD_2	PGG_2	PGI_2	PGE_2
0.61 ± 0.12	1.71 ± 0.37	1.91 ± 0.38	1.0
$n=10$	$n=7$	$n=12$	
$P<0.05$	n.s.	$P<0.05$	

TABLE III Vaso-depressor effect of a standard dose ($10 \mu\text{g}$) of PGE_2 , PGG_2 and PGI_2 given either in the jugular vein (i.v.) or in the aortic arch (i.a.) in cats. D is presented as percent fall in blood pressure and given as means \pm S.E. (N) where N is number of experiments.

	PGE_2	PGG_2	PGI_2
i.v.	23.2 ± 4.0 (8)	27.6 ± 4.4 (7)	34.3 ± 3.4 (10)
i.a.	31.0 ± 2.4 (3)	27.6 ± 4.1 (3)	31.6 ± 2.0 (3)
	$P<0.05$	n.s.	n.s.

PGI_{2a} was purely depressor. Independently of whether PGI_{2a} was pressor or depressor it consistently caused a slight and shortlasting decrease in the heart rate.

In some experiments administration of PGG_2 or PGI_2 was repeated after incubation of the compound in 0.9% NaCl or blood for 20 min at 37°C . This treatment almost abolished the effect of the compound on pulmonary arterial pressure and tracheal insufflation pressure and it reduced the effect on systemic arterial pressure, i.e. resultant effects closely resembling those of PGE_2 (Fig. 2).

In order to get more insight in effects of the endoperoxides *per se* the stable endoperoxide

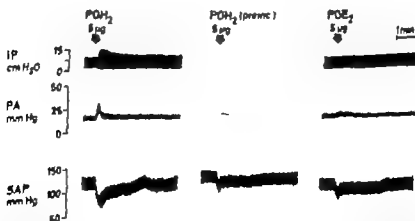
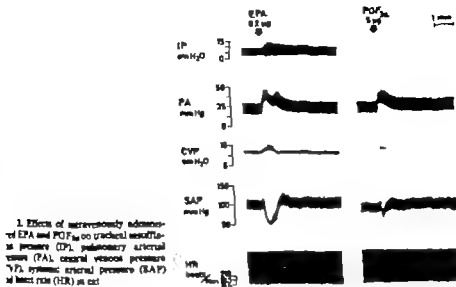


Fig. 2. Effects of intravenously administered PGI_2 , PGI_2 preincubated in 0.9% NaCl for 20 min at 37°C , and PGE_2 on tracheal insufflation pressure (IP), pulmonary arterial pressure (PA) and systemic arterial pressure (SAP) in cat.



alogous EPA was used. EPA doses of the order of 0.1–0.5 μ g sufficed to markedly increase tracheal insufflation pressure and pulmonary arterial pressure. In the latter case a two peak response was often observed, suggesting recirculation of intact EPA (Fig. 3). Relative to PGF_{2α}, EPA was 30–40 times more potent on tracheal insufflation pressure and pulmonary arterial pressure (Table I). As with PGF_{2α}, EPA produced mixed effects on systemic arterial pressure. The most prevalent response was an initial drop followed by a moderate increase, although pure depressor or pressor responses were also seen. The effect of EPA on the heart rate was consistently that of a slight decrease.

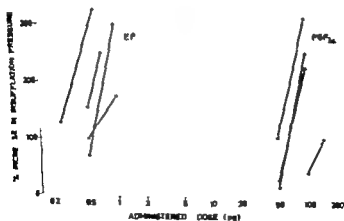


Fig. 4. Dose-response relations for intravenously administered PGF_{2α} and EPA on tracheal insufflation pressure in 4 guinea pigs.

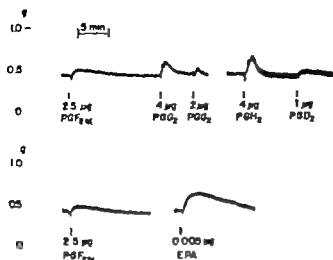


Fig. 5. Contractile effects of $\text{PGF}_{2\alpha}$, PGG_2 , PGH , PGD_2 and EPA on a superfused human bronchial strip.

Guinea pig experiments

Previously it has been shown that the endoperoxides are 8–11 times more potent than $\text{PGF}_{2\alpha}$ in eliciting an increase in tracheal insufflation pressure in guinea pigs (Hasthorp *et al.* 1975a). In the present expts. the endoperoxides were replaced by EPA and its effect on tracheal insufflation pressure was compared with that of $\text{PGF}_{2\alpha}$. Both compounds increased the tracheal insufflation pressure, but EPA was considerably more potent than $\text{PGF}_{2\alpha}$ (Fig. 4). Calculating the relative activity revealed that EPA was 167 ± 41 (mean \pm S.E., $n=4$) times more potent than $\text{PGF}_{2\alpha}$ in this respect. The effect of EPA on the cardiovascular system was similar to that of $\text{PGF}_{2\alpha}$, i.e. a transient fall in systemic arterial blood pressure and bradycardia, followed by a more longlasting rise in pressure and tachycardia.

Human bronchi in vitro

In Fig. 5 is illustrated the contractile effect of PGD_2 , $\text{PGF}_{2\alpha}$, PGG_2 , PGH and EPA on isolated human bronchi, superfused with Tyrode at a rate of 1.5 ml/min. PGD_2 , PGG_2 and PGH were approximately as potent bronchoconstrictors as $\text{PGF}_{2\alpha}$, whereas EPA was considerably more active in this respect. The relative contractile effects of the different compounds are shown in Table IV.

In other expts. human bronchi were incubated in 5 ml Tyrode, and dose response curves for $\text{PGF}_{2\alpha}$ and EPA were determined by stepwise raising the concentrations of the compounds in the bath. Approximately parallel dose-response curves were obtained, but EPA was 92 ± 45 (mean \pm S.E., $n=7$) times more active than $\text{PGF}_{2\alpha}$.

TABLE IV Relative contractile effects of PGD_2 , PGG_2 , PGH , EPA and $\text{PGF}_{2\alpha}$ on isolated human bronchi. Mean values \pm S.E. Figures within brackets—number of expts.

PGD_2	PGG_2	PGH	EPA	$\text{PGF}_{2\alpha}$
1.2 ± 0.2 ($n=3$) n.s.	1.2 ± 0.4 ($n=4$) n.s.	1.0 ± 0.8 ($n=4$) n.s.	369 ± 146 ($n=5$) $P < 0.01$	1.0

Discussion

In the present study PGG and PGH were found to increase tracheal insufflation pressure significantly more than did PGF_{α_2} , which is in harmony with previous observations in guinea pig (Hamberg *et al.* 1975 a). The endoperoxides also increased pulmonary arterial pressure and they were of the same potency as PGF_{α_2} in this respect. Ånggård and Persson (1963) reported that PGF_{α_2} increased both tracheal insufflation and right ventricular pressures in cats and attributed the bronchial effect of the compound to constriction of bronchial smooth muscle or pulmonary congestion or a combination of factors. However because the effect of PGF_{α_2} and endoperoxides on pulmonary arterial pressure is similar the relatively higher potency of the endoperoxides on tracheal insufflation pressure found in the present study is hard to explain in terms of pulmonary congestion. Moreover while PGF_{α_2} caused bradycardia the endoperoxides consistently caused an increase in heart rate. Therefore, the endoperoxides should be considered not bronchoconstrictors *in vivo* just as they have been shown to be in different *in vitro* tests (cf Hamberg *et al.* 1975 a).

PGD_2 , PGE_2 , PGG and PGH depressed systemic arterial blood pressure and meanwhile caused a moderate rise in heart rate, presumably secondary to the fall in blood pressure. These compounds are depressors also in the rat (Armstrong *et al.* 1976), whereas in the guinea pig PGD_2 and PGE_2 are depressors and the endoperoxides cause a triphasic change in blood pressure (Hedqvist *et al.* 1974 Hamberg *et al.* 1975 a). PGH was significantly more active than PGE_2 when given by the i.v. route. This difference in activity was annulled when PGH was preincubated in saline or when the two compounds were given i.a. Moreover PGH was equally active on i.v. and i.a. administration, and PGE_2 more active when given i.a. than i.v. The difference in activity between the two compounds upon i.v. administration could be explained by PGE_2 being partly metabolized during the passage through the lung circulation (Ferreira and Vane 1967), and PGH leaving the lung circulation either intact or as an active metabolite, which could be PGE_2 but not PGD_2 or PGF_{α_2} . Admittedly PGH caused a rise in pulmonary arterial and central venous pressures but the short duration of these effects makes it unlikely that pulmonary congestion should have affected significantly the vasodepressor activity of the compound.

The relative potency of PGE_2 and PGH as vasodepressor agents has been studied also in the rat (Armstrong *et al.* 1976). In this animal species PGE_2 was found to be 6 times more effective than PGH in reducing systemic arterial blood pressure upon i.v. administration, whereas the two compounds were approximately equactive when given i.v. Species differences may account for the dissimilar results in rat and cat.

Because the endoperoxides may be rapidly transformed into other compounds, chiefly PGE_2 , PGF_{α_2} and PGD_2 (Hamberg and Samuelsson 1973 Nugteren and Hazebroek 1973) but also thromboxanes (Hamberg *et al.* 1975 b) their bronchial and cardiovascular effects were compared with those of the stable endoperoxide analogue EPA. EPA was found to be 30-40 times more potent than PGG or PGH in increasing bronchial and pulmonary vascular resistances in the cat. In the guinea pig EPA was near 200 times more potent than PGF_{α_2} in causing an increase in tracheal insufflation pressure. The difference in

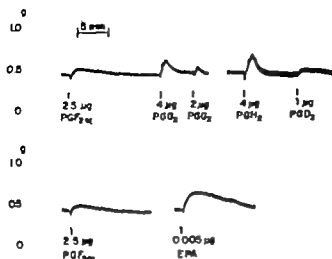


Fig. 5 Contractile effects of $\text{PGF}_{2\alpha}$, PGG_2 , PGH_2 , PGD_2 and EPA on superfused human bronchial strip.

Guinea pig experiments

Previously it has been shown that the endoperoxides are 8–11 times more potent than $\text{PGF}_{2\alpha}$ in eliciting an increase in tracheal insufflation pressure in guinea pigs (Hamberg *et al.* 1975a). In the present expts. the endoperoxides were replaced by EPA and its effect on tracheal insufflation pressure was compared with that of $\text{PGF}_{2\alpha}$. Both compounds increased the tracheal insufflation pressure, but EPA was considerably more potent than $\text{PGF}_{2\alpha}$ (Fig. 4). Calculating the relative activity revealed that EPA was 187 ± 42 (mean \pm S.E., $n=4$) times more potent than $\text{PGF}_{2\alpha}$ in this respect. The effect of EPA on the cardiovascular system was similar to that of $\text{PGF}_{2\alpha}$, i.e. a transient fall in systemic arterial blood pressure and bradycardia, followed by a more longlasting rise in pressure and tachycardia.

Human bronchi in vitro

In Fig. 5 is illustrated the contractile effect of PGD_2 , $\text{PGF}_{2\alpha}$, PGG_2 , PGH_2 and EPA on isolated human bronchi, superfused with Tyrode at a rate of 1.5 ml/min. PGD_2 , PGG_2 and PGH_2 were approximately as potent bronchoconstrictors as $\text{PGF}_{2\alpha}$, whereas EPA was considerably more active in this respect. The relative contractile effects of the different compounds are shown in Table IV.

In other expts. human bronchi were incubated in 5 ml Tyrode, and dose-response curves for $\text{PGF}_{2\alpha}$ and EPA were determined by stepwise raising the concentrations of the compounds in the bath. Approximately parallel dose-response curves were obtained, but EPA was 97 ± 45 (mean \pm S.E., $n=7$) times more active than $\text{PGF}_{2\alpha}$.

TABLE IV Relative contractile effects of PGD_2 , PGG_2 , PGH_2 , EPA and $\text{PGF}_{2\alpha}$ on isolated human bronchi. Mean values \pm S.E. Figures within brackets: number of expts.

PGD_2	PGG_2	PGH_2	EPA	$\text{PGF}_{2\alpha}$
1.2 ± 0.2 ($n=3$) n.s.	1.2 ± 0.4 ($n=4$) n.s.	2.2 ± 0.8 ($n=4$) n.s.	569 ± 146 ($n=5$) $P < 0.01$	1.0

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activity should be compared with our previous report that the endoperoxides are 1-4 times more potent than $\text{PGF}_{2\alpha}$ in this respect (Hamberg *et al.* 1975a). Recently still although less dramatic effects of EPA have been shown in the dog, inasmuch EPA is 4-8 times more potent than $\text{PGF}_{2\alpha}$ in causing a decrease in lung compliance and increase in lung resistance (Wasserman 1976). Furthermore another endoperoxide analog resembling EPA was approximately 10 times more active than $\text{PGF}_{2\alpha}$ in increasing arterial pressure in canine lung lobes perfused with blood or dextran (Kadowitz and Ivers 1977). The effects obtained with EPA therefore seem to indicate that endogenously formed endoperoxides are more potent on pulmonary vascular and bronchial smooth muscle than is obvious from their exogenous application. However in the cat EPA produces some cardiovascular effects, which differed from those obtained with endoperoxide: biphasic changes in blood pressure and bradycardia rather than fall in pressure and tachycardia. Moreover in the dog EPA decreases and PGH increases blood flow in the mesenteric artery (Chaplin *et al.* 1977). Therefore the possibility should not be overlooked that also in the pulmonary circulation EPA produces effects that cannot be accounted for by endoperoxides.

PGD_2 , $\text{PGF}_{2\alpha}$, PGG_2 , PGH_2 and EPA were all found to contract isolated human bronchi. PGD_2 , $\text{PGF}_{2\alpha}$, PGG_2 and PGH_2 were approximately equiactive, whereas EI demonstrated a 90-190 times higher potency. Previously it has been shown that PGD_2 is 5 times and the endoperoxides 8-9 times more potent than $\text{PGF}_{2\alpha}$ in eliciting contraction of isolated guinea pig trachea (Hamberg *et al.* 1975a). The relatively lower activity of endoperoxides and PGD_2 on human bronchi might reflect different patterns of reaction. However the difference could also be due to a more rapid conversion of endoperoxides into their end products in human bronchi than in guinea pig trachea. Consistent with latter view is the remarkable bronchoconstrictive effect of EPA compared with the endoperoxides.

Primary prostaglandins have been implicated in normal and disordered lung (especially bronchial asthma) (cf. Mathé *et al.* 1977). Recent studies have shown, however, that PGG_2 and PGH_2 are 8-11 times and thromboxane A₂ 500 times more potent than $\text{PGF}_{2\alpha}$ in increasing tracheobronchial insufflation pressure in guinea pig (Hamberg *et al.* 1977; Svensson *et al.* 1977). The present data demonstrating marked pulmonary actions of endoperoxides and an endoperoxide analogue in cats and guinea pigs *in vivo* and human bronchi *in vitro* add further weight to the concept that a significant proportion of biological effects occurring on activation of prostaglandin synthetase can be accounted for by endoperoxides and/or thromboxane A₂. Since these compounds are all potent bronchoconstrictors a role for the E prostaglandins with regard to airway patency will seem difficult to recognize. It is conceivable, however, that conversion of endoperoxides into thromboxane A₂ occurs only under certain conditions, and that the biological action of endoperoxides *per se* is balanced by their half life normally being short in comparison with that of PGE_2 .

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The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons

By

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Abstract

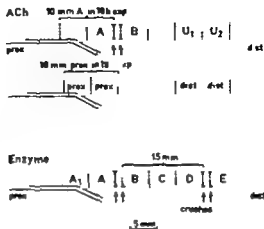
DAHLSTRÖM A., P-O HEIWall, S BÖÖJ and A-G DAHLÖF *The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons* Acta physiol. scand. 1978. 103 308-319

The effect of supraspinal impulse activity upon the intra-axonal transport of acetylcholine (ACh), AChE and choline acetyltransferase (CAT) in rat sciatic nerve has been studied. A decreased impulse activity was obtained by spinal cord transection (SCT) in the thoracic region III 2, 6 days or 12 days before killing the rats. A decreased neuronal activity was obtained by exercising the rats on a commercial rodent treadmill a couple of hours per day for 14 days. The amounts of substances which had accumulated in the sciatic nerve segments relative to nerve crush performed III or 12 h earlier were used to calculate the intra-axonal transport. The amounts of proximo-distally transported ACh decreased markedly with time after the SCT while the proximo-distal transport of AChE-activity increased. Physical exercise appeared to increase ACh-transport. Thus, it is suggested that motor periphery from supraspinal centres may regulate intra-axonal transport from the cell body of motor neurons into their axons.

Key words. Intra-axonal transport, rat motor neurons, acetylcholine, cholinergic enzymes, spinal cord transection

In motor nerves of mammals a proximo-distal, intra-axonal transport of various substances takes place. Thus, labelled proteins have been found to be transported at rates varying between 2-3 mm/day and 400 mm/day (cf. Ochs and Johnson 1969). The slow phase of transport mainly carries soluble proteins and microtubular and filamentous proteins while preferentially membrane-bound material is moving with the more rapid rates of transport (e.g. Sabri and Ochs 1973). In agreement with this the soluble enzyme choline acetyltransferase (CAT) in rat motor nerves appears to be transported with the slow phase (Saunders *et al.* 1973) while the acetylcholinesterase (AChE) which is membrane-bound (Kása *et al.* 1973), is transported at rapid rates (e.g. Lublířská and Niemlerko 1971, Ranish and Ochs 1972).

Also the transmitter in motor nerves, acetylcholine (ACh), appears to be transported intra-axonally at rates of about 120 mm/day in the rat sciatic nerve (e.g. Dahlström *et al.*



1. Schematic illustration of the mode of transport of the rat sciatic nerve, relative to each nerve. Each crush was consisted of sites separated by 1 mm nerve.

Ha). It is not known in what compartment this transportable ACh is located, but due to the rather rapid rate of transport it is likely that this ACh (about 15–20% of total axonal ACh) may be bound within a membrane-limited structure (see discussion Dahlström *et al.* 1974 b).

Changes in impulse activity of a neuron can influence the amounts of transported ACh, whilst the rate of transport seems to be unaltered (*cf.* reviews by Dahlström *et al.* 1974, Hedgpeth 1975). Thus, increased impulse activity in adrenergic nerves causes an increase in the amounts of transported noradrenaline and dopamine β -hydroxylase in the axons (Ken and McLean 1975). In the present study we have therefore investigated the effect of both decreased and increased impulse activity on the intra-axonal transport of ACh, CAT and AChE in rat sciatic nerve.

Materials and methods

Male rats (200–250 g) of both sexes were used in all experiments. Decreased impulse activity in the sciatic nerve was obtained by sectioning the spinal cord at the lower thoracic level 18 h, 6 days or 20 days prior to surgery. All surgical procedures were performed under ether anaesthesia. The bladder of the spinal cord (SCT) material was emptied by exterior manipulations twice daily. This was found to be necessary since all SCT animals showed marked spinal haemorrhage, leading to plug the stretch of the bladder was not emptied regularly. This haemorrhage disappeared after a couple of days. It is easier to keep female rats healthy during longer postoperative periods, hence was the reason for using also female rats, especially for the 20 days SCT study. Control animals were handled twice daily and housed in the same room as the SCT animals.

An increased impulse activity was obtained by exercising the rats 2–3 h daily for 14 days in a running wheel treadmill (Quinton Instruments, Seattle, Wash.). Controls were allowed to stay in the treadmill for an equivalent period of time but without moving the track. The exercise as used at the beginning of the period and gradually increased in speed and duration (*cf.* Barnard and Peter 1971). Individual rats showed large variation in exercise tolerance, some had to be excluded from the experiment at an early stage due to poor capacity.

12 h or 14 h (for exercise exp.) prior to sacrifice about half of the animals in each group were reoperated under ether anaesthesia. The sciatic nerves bilaterally were exposed and nerve crushes (a.m. Laubrock 1977) were made between a glass rod (0.5 mm) and silk suture (3/0). For ACh assays the nerves were crushed at both levels only. The crush is included 2 crushes separated by 1–2 mm. 12 h after crushing 5 mm segments just proximal (A) and distal (B) to the site of

The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons

By

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Abstract

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The effect of supraspinal impulse activity upon the intra-axonal transport of acetylcholine (ACh), AChE and choline acetyltransferase (CAT) in rat sciatic nerve has been studied. A decussate impulse activity was obtained by spinal cord transection (SCT) in the thoracic region III b, 6 days before killing the rats. An increased neuronal activity was obtained by exercising the rats on a commercial rodent treadmill: couple of hours per day for 14 days. The amounts of substances which had accumulated in the sciatic nerve segments relative to a nerve crush performed 12 or 18 h earlier were used to calculate the intra-axonal transport. The amount of proximo-distally transported ACh decreased markedly with time after the SCT while the proximo-distal transport of AChE-activity increased. Physical exercise appeared to increase ACh-transport. Thus, input to motor pericarya from supraspinal centres may regulate intra-axonal transport from the cell body of motor neurons into their axons.

Key words. Intra-axonal transport, rat motor neurons, acetylcholine, cholinergic enzymes, spinal cord transection.

In motor nerves of mammals a proximo-distal, intra-axonal transport of various substances takes place. Thus, labelled proteins have been found to be transported at rates varying between 2-3 mm/day and 400 mm/day (cf. Ochs and Johnson 1969). The slow phase of transport mainly carries soluble proteins and microtubular and filamentous proteins while preferentially membrane bound material is moving with the more rapid rates of transport (e.g. Sabat and Ochs 1973). In agreement with this the soluble enzyme choline acetyltransferase (CAT) in rat motor nerves appears to be transported with the slow phase (Saunders *et al.* 1973) while the acetylcholinesterase (AChE), which is membrane-bound (Kása *et al.* 1973), is transported at rapid rates (e.g. Lubitska and Niemierko 1971, Ranish and Ochs 1977).

Also the transmitter in motor nerves, acetylcholine (ACh), appears to be transported intra-axonally at rates of about 120 mm/day in the rat sciatic nerve (e.g. Dahlström *et al.*

CAT act. by % of control
 uncrushed 5 mm nerve

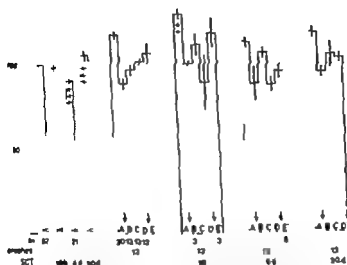


Fig. 2. CAT-activity expressed as percent of control uncrushed nerve in distal sciatic nerve at various sites following spinal cord transection (SCT). Arrows indicate site of transection (see Fig. 1). Mean \pm S.E. are given: 100 = 996 ± 83 pmol of ACh formed/hour/5 mm nerve. Indicated by asterisks (*) indicates different from control uncrushed nerve, $p < 0.05$.

served after SCT although a tendency towards lower values were seen in 18 h and 20 d SCT-operated animals. In all groups, the distal 5 mm segment appeared to contain more ACh than the proximal segment.

In the 12 h crushed nerves of the control group the segment A contained 139 ± 4.4 pmol of ACh. After SCT the ACh in the A-segment was only 107 ± 6.1 pmol in 18 h operated animals and 90.9 ± 9.9 pmol 20 days after the spinal cord operation (Fig. 2). In the B-segment, just distal to the crushes, the ACh-content was somewhat lower in 18 h and 20 d SCT-nerve, but normal or possibly supranormal in 20 days operated rats. In the C-segment, which were assayed for ACh to give some information on the "transportable" fraction of ACh, the ACh content was always somewhat lower (7%, 9%, 16% and 20% in 0 to 20 d SCT animals, respectively Fig. 2) than the corresponding control piece in uncrushed nerves (distal), but a significant decrease was obtained only in the 20 d SCT operated animals.

The amount of ACh transported to the A-segment from more proximal sites in the nerve by axonal transport can be obtained by the following calculation. $ACh_A = ACh_{A, uncrushed} - ACh_{A, crushed}$. Since the B-segment contains $ACh_{B, uncrushed} - ACh_{B, crushed}$ (Feldberg 1943), the calculation is reduced to $ACh_A = ACh_{B, uncrushed} - ACh_{B, crushed}$. This is indicated in Fig. 6. It can be seen that the rapid initial decrease in transported ACh is about 17% (18 h) and by 20 days the decrease was about 80% (Fig. 6).

CAT in uncrushed nerves of control animals no difference in CAT-activity was observed along the nerve in the various segments A-E. The CAT-activity in the uncrushed nerves

150 pmol ACh / 5 mm nerve

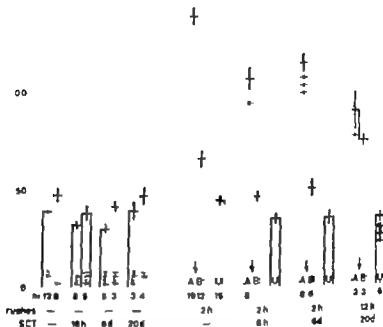


Fig. 2. The ACh content of intact or 12 h crushed rat sciatic nerve at various times after spinal cord transection (SCT) at the thoracic level V-nervous segments as indicated in Fig. 1. Arrow indicates the crush. Mean \pm S.E. are given. * different from control A-segment at $p < 0.005$. C, D, E different from dist. uncrushed nerve of 20 days SCT rat at $p < 0.005$.

crush were dissected, as well as one or two 5 mm segments (10–20 mm distal to the site of the crush (U + U) (Fig. 1). 5 mm segments from comparative levels of uncrushed nerves were dissected from rats which were not re-operated. In the physical exercise experiment the A segment was 10 mm instead of 5 mm and the nerves were dissected out 18 h after the crush operation (Fig. 1). The animals were made unconscious by a blow on the head, the nerves were taken out and immediately put on an ice-cold plate and cooled prior to cutting. 2–6 nerve segments were pooled in each sample. ACh was extracted according to Macintosh and Perry (1950) and assayed on guinea pig ileum preparation (Blaber and Cuthbert 1961). An isometric muscle transducer (Model 33-03-1693 Gould Inc. Instruments Syst. Div. Cleveland, Ohio) as well as a recorder registered the contractions of the gut and recordings of the contractions was made on a potentiometer recorder (Goerz Servogor 5 RE 341). Schild's four-point assay technique (Schild 1942) was used in calculating the results. An antihistamine (Mepyramine[®]) was added to the Krebs solutions used. The activity of the samples was identified as ACh by inactivation with purified AChE (Worthington Biochemical Corp., Freehold NJ) and by complete inhibition by tropic acid. The response of the gut is standard and sample. The activity measured was thus ACh-like but will be referred to as ACh in the following.

For assays of CAT and AChE the sciatic nerves bilaterally were crushed both at the high level and 15 mm more distally. Each site of crushing consisted of two crushes with 1–2 mm distance between the two crushes (Fig. 1). 12 h or 18 h (if exercise points) later the rats were killed by decapitation. The nerves were dissected out, placed on a cooled plate and cut into 5 mm segments relative to the two sites of crushes. Comparative sections from uncrushed nerve were also dissected and assayed for enzyme activities. CAT and AChE was assayed radiochemically in the same 5 mm segment according to the method of Tóček (1974), with some modifications.

Results

Decreased impulse activity

ACh. The level of ACh in the various nerve segments of control animals and SCT animals is shown in Fig. 2. In uncrushed nerves no significant changes in ACh-content were

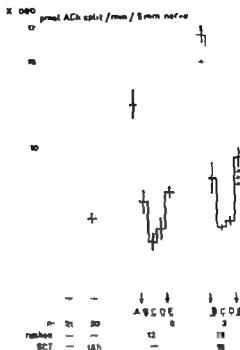


Fig. 6. AChE-activity in the sciatic nerve of control or 12 h speed-learned (SCT) rats. Asterisks indicate use of crushes (see text). Mean \pm S.E. are given. Results from particular experiments indicate different from control, $p < 0.05$ indicates different from control uncrushed nerve, $p < 0.01$ indicates different from control A and B segments, respectively $p < 0.005$.

amount of enzyme activity which was transported to the A-segment during 12 h (A minus uncrushed) increased after SCT as shown in Fig. 6.

Increased enzyme activity

ACh in uncrushed nerves there was no marked difference in ACh content after physical training, as compared to control animals. In the A-segment, however a 20% increase was seen in the exercised animals. The amount of ACh transported to the A-segment

$$\left(A_{\text{prox}} - \left(B_{\text{prox}} \right) \right)$$

was increased from 117.5 pmol to 142 pmol, i.e. by 21%. In the control material the "transportable" fraction of ACh

$$\left(\frac{\text{dist. uncrushed}}{\text{dist. uncrushed}} \right)$$

in 21% in the exercised animals, this calculation could not be done due to few observations and large variance (Fig. 7).

After 12 h of physical training no significant changes in enzyme activity was seen in uncrushed nerves after physical training and no difference was observed between the right and left nerves. In the 12 h crushed nerves the only segment which differed from control after training was the

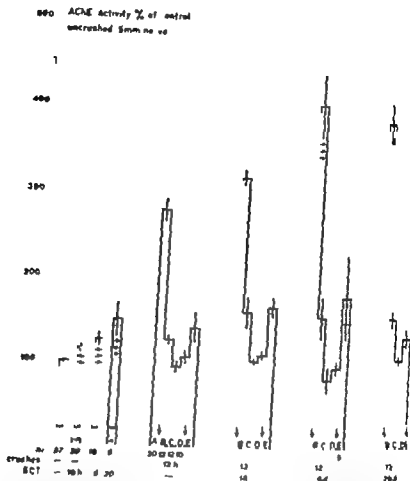


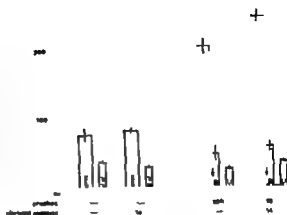
Fig. 4 AChE-activity in rat sciatic nerve at various times after spinal cord transection (SCT). Asterisks indicate site of crushes (see Fig. 3). Mean \pm S.E. are given. 100 = 4.22 ± 1.57 pmol of ACh split/min/mg nerve. * indicates different from respective control nerve (un-crushed or 1 h crushed, segment A) at $p < 0.005$.

was somewhat decreased at 6 days after SCT and somewhat increased by 20 days. The A-segment of 12 h crushed nerves contained significantly more CAT-activity in the 12 h SCT animals than in the controls. In the section between the two sites of crushes the CAT activity was redistributed the middle piece (c) often containing the highest enzyme activity (Fig. 3).

AChE. In uncrushed nerves of the control group no significant difference in AChE activity between the various segments A-E could be noticed. Therefore all observations from uncrushed nerves were taken together as indicated in Fig. 4. In uncrushed nerves the AChE-activity gradually increased with time after SCT reaching 150% of control 20 days after SCT (Fig. 4). The A-segment of 12 h crushed nerves likewise showed an increase in enzyme activity with time after SCT reaching maximum after 6 days. However already 18 h after SCT the A segment contained significantly increased AChE-activity when compared to the control group of that particular experimental occasion (Fig. 5). The C-segment contained the lowest enzyme level in all groups of animals and was about 5-15, 10, 35 and 30% lower than in uncrushed nerves of the respective groups (Fig. 4-5). The

200 pmol ACh / 10 mm nerve

Fig. 7 The ACh-content of uncrushed or 10% crushed sciatic nerve of rats, increased for 14 days. Mean \pm S.E. of the ACh content of 5 or 10 mm nerve segments (indicated by different bars) relative to the crush level (see Fig. 1). * indicates different from uncrushed nerve, distal; ** indicates different from control A-segment, 10%.



The subcellular distribution of CAT and AChE in vivo

The subcellular intra-axonal distribution of the two enzymes appear to agree very well with that suggested for rabbit sciatic nerve (Tudek 1975). In rabbit sciatic nerve, AChE is concentrated preferentially in the proximal 2 mm segment nearest a ligation while the maximal increase in CAT activity was observed in the segment 2-4 mm proximal to the ligation. This differential distribution may well be explained by the fact that AChE is bound in the axon to the sER which is transported at a rapid rate. This organelle thus occupies the space just above the ligation, displacing the axoplasm, containing the soluble CAT further away in a proximal direction. In the present study we dissected 5 mm segments, also proximal to the high crush levels, and have therefore most likely included both

200 pmol ACh formed / 10 mm nerve



Fig. 8 CAT-activity in uncrushed or 10% crushed sciatic nerve of control and crushed rats. Arrows indicate sites of crushes. Mean \pm S.E. are given. Segments were dissected as shown in Fig. 1 and cores 4 & 5 from control A-A pairs (taken together), $p < 0.05$. *** indicates different from control E-segments.

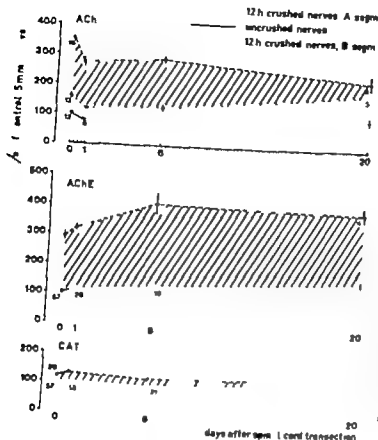


Fig. 6. Changes, caused by spinal cord transection, in the amount of proximo-distally transported substances in the rat sciatic nerve. The striped areas show the amounts transported to the A segment, proximal to the hip crush, during 12 h after the crush operation. Calculations performed as indicated in the text. Mean \pm S.E. are given, small boxes show number of observations.

E-segment distal to the distal crush level (Fig. 8). However when segments A and A were taken together a small but significant decrease was observed after physical exercise AChE. No changes in enzyme activity was observed in any nerve segment after training.

The methods used

The biological method to assay ACh used in this study has been discussed in a previous paper (Dahlström *et al* 1974a). There is little doubt that the assayed activity really represented ACh. Support for this view is obtained by results from chemical identification of ACh in rat sciatic nerve (Ulin *et al* 1976) which gave similar results to ours concerning the amount of ACh per cm uncrushed sciatic nerve.

Discussion

The enzyme methods used have been discussed by Tuček (1974). The activities of CAT and AChE in uncrushed rat sciatic nerve compare well with results of other investigators using other methods. Thus, CAT activity in rat sciatic nerve was earlier reported to be between 7–10 μ mol of ACh formed/hr/g (Fonnum 1969; Saunders *et al* 1973). In the present study CAT-activity of uncrushed normal nerve was between 8–10 μ mol of ACh formed/h/g, the weight of 5 mm nerve being about 5 mg. The AChE-activity of rat sciatic nerve was earlier found to be 56 μ mol of ACh split/h/g (Kása *et al* 1973) while our own value in the present study was about 60 μ mol of ACh split/h/g.

raise the membrane-affinity of the CAT while the enzyme is mainly soluble during axonal conditions (Fonnum 1967, 1968).

variations in axonal transport by SCT

In this study we have tried to estimate the amount of the various substances that were transported with proximo-distal transport down the nerve during the 1 h crush period. For this the amount may be calculated according to the formula $ACh_A - ACh_B = ACh_{transported}$. The ACh in segment A represents the content in uncrushed nerve plus the amount carried by axonal transport plus locally synthesized ACh, due to the axonal transport (cf. Feldberg 1943). The ACh in segment B probably represents the amount in the crushed prox. segment + the locally synthesized ACh (Dahlstrom *et al.* 1974 a). As can be seen in Fig. 2 the amount of ACh in segment B varied in the various groups of SCT animals. The reason for this may be either changes in $ACh_{transported}$ or changes in ACh_{local} . It is difficult to imagine that SCT can influence the local ACh synthesis during crushing. On the other hand, the $ACh_{transported}$ was decreased in 18 h and 6 days SCT animals (prox. segments, Fig. 2), when the ACh was below control levels, while at 20 days after SCT the ACh in uncrushed proximal segments was again in the control range, it was the ACh_B of the 20 days SCT group. Therefore, variations in $ACh_{transported}$ were probably due to variations in ACh_{local} .

As can be seen in Fig. 6, the amount of ACh carried by axonal transport to the A segment decreased markedly with time after SCT. The amount of transported AChE, on the other hand, calculated according to the formula $AChE_A - AChE_{transported}$ (no local synthesis involved) was found to increase after SCT (Fig. 6, middle). A possible decrease in amounts of transported CAT may have occurred after SCT (Fig. 6, bottom).

It is very interesting that a decreased impulse activity has so pronounced effects on axonal transport of ACh and AChE as has been demonstrated in this study. Previous studies have shown little or no effect of preganglionic denervation on intra-axonal transport of NA and amine granules in adrenergic neurons (Geffen and Rush 1968, Banks *et al.* 1971) while an increased activity gave increases in amount of transported amine granules (Keen and McLean 1975).

Effects of physical exercise

After 14 days of daily physical exercise, which presumably caused a net increase in the impulse activity of the motor neurons of the hindlimb, the amounts of ACh accumulated in the 1 cm long A-segment was clearly increased. The transported amounts appeared to be about 25% higher than in the control group. The transportable fraction was about 45%

$$\left(\frac{\text{dist. uncrushed}}{\text{dist. uncrushed}} \cdot U \right)$$

in the control group, but appeared less in the exercised group although no calculations could be performed due to few observations. No clear changes were seen in the AChE levels, except that the proportion of proximodistally transported enzyme may have been higher after exercise, as indicated by the pattern of redistribution in the segment between

the segment with high AChE-activity and high CAT-activity in our estimations on row transport

In the 12 h double crush expts., both enzymes were redistributed within the segment isolated by the high and low crush sites, but in a very different manner suggesting a different subcellular distribution of the two enzymes

a) The AChE had moved towards both ends, leaving the middle segment (c) with the lowest activity. The decrease from normal levels in this segment probably indicates the transportable fraction of AChE in the nerve (cf. Lubrińska and Niemierko 1971, Fonnum *et al.* 1973, Tuček 1975). In all experimental groups in this study there was probably a decrease below uncrushed levels in this segment, but the difference was statistically significant only for 6 days and 30 d SCT rats. In these two groups the decrease in the C-segment was 35 and 30%, respectively. In most experimental groups the B-segment contained twice or more the AChE levels of the D-segment, indicating that in the boole rat sciatic nerve a larger proportion of AChE may move in the retrograde direction than in the proximo-distal direction. This is in contrast to the findings by Lubrińska and Niemierko (1971) in the dog peroneal nerve and by Fonnum *et al.* (1973) in the rabbit vagus and hypoglossal nerves, where the larger proportion of transportable AChE moved in the proximo-distal direction in isolated nerve segments. However, Tuček (1975) found in rabbit sciatic nerve more AChE accumulating in the retrograde than in the anterograde direction. One may wonder then why in our results the A-segment accumulated so much more esterase-activity than the E-segment, because the reverse should be the case if retrograde transport was most prominent. Since two crushes were performed at each side of the crush, contamination from A to B and from E to D may be ruled out. One cause for the comparatively low enzyme level in segment E may be that the transportable fraction of AChE was exhausted at an early stage after crushing. The nerve length between the distal crush site and the motor endplates may be estimated to be only about 15 mm, and the transportable AChE-fraction of that 15 mm nerve would approximately correspond to the increase above uncrushed levels that was seen in the E-segment in e.g. the 6 day SCT nerves (Fig. 4). The A segment, on the other hand, was continuous with the long proximal nerve and with the cell bodies, so there was no limit to the anterograde accumulation of AChE in this segment.

b) The CAT-activity was in most cases higher in the middle C-segment of the isolated nerve than in the B and D segments. This may be due to the same displacement of axoplasm from the ends of a crushed fibre as occurred in the ends of rabbit sciatic nerves after ligation (Tuček 1975). Thus, it appears that in rat sciatic nerve the CAT is soluble and clearly separated in subcellular localization from AChE. In rabbit hypoglossal and vagal nerves the CAT appeared to be partly membrane affiliated since a decrease in the middle and increase towards both ends of an isolated nerve segment occurred (see Fig. 1 in Fonnum *et al.* 1973). The results in the present study indicate that CAT is transported at a slow rate (a few mm/d) in agreement with the observation in rabbit sciatic nerve (Tuček 1975), but in contrast to the results from rabbit vagus and hypoglossal nerves (Fonnum *et al.* 1973). These diverging results are difficult to explain, but possible reasons may be e.g. variable osmolarity in various nerves, since it has been shown that hypotonic conditions

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the two crush sites (D-segment having slightly higher AChE activity than the B-segment). The CAT was in main unchanged, but if the two segments above the high crush (A and B) were calculated together a small but significant decrease was observed after crush.

The changes caused by increased impulse activity are comparatively small when compared to the changes induced by SCT. It may be that other time intervals after crush exercise could have induced more pronounced changes. Shorter intervals will be tested in a following study. However an increase in the transported amount of ACh was present. Since increased stimulation of a perikaryon is known to stimulate the protein synthesis (Hydén 1960), this observation was not unexpected. A decrease in the transport of CAT was, however, not in line with the increase in ACh transport.

In the present study we have demonstrated that the impulse activity in motor nerves monitored by supra-spinal centres via descending axons, may regulate the synthesis and proximo-distal intra-axonal transport of what is probably ACh-storing organelles and AChE and possibly also CAT in motor nerves. If alterations may occur in the intra-axonal transport of these substances, it is likely that also transport of other substances, e.g. hypothetical trophic substances (cf. Gutmann 1964, Korr *et al.* 1967, Ochs 1974) may occur following e.g. trauma of the spinal cord. Therefore, the atrophic changes observed in muscles of paralyzed individuals may be due not only to disuse of the limb but also to an altered delivery of trophic substances from the nerve to the muscles.

It may be speculated which descending systems that are most responsible for the control of the motor perikaryon with respect to the intra-axonal dynamics of ACh and the related enzymes. Unmyelinated axons may be involved since long-term proton irradiation of the spinal cord causing a severe demyelination of the white matter did not affect the transport of ACh, AChE or CAT (Heiwall *et al.* 1978) to any large degree. The possibility that descending monoaminergic neurons are involved will be tested in a forthcoming paper.

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Since then been published for many membrane systems (for references, see Ågren and Ronquist 1976 a). Endogenous protein kinase was also demonstrated at the cell surface of both rat glioma and glioma cells in culture (Ågren and Ronquist 1974). This observation was also shown to be valid for also rat C-6 glioma cells (Schlaeger and Köhler 1976) as well as 3T3 cells (Mastro and Rozengurt 1976). Furthermore Ehrlich cells, glioma cells and glioma cells were able to phosphorylate exogenous acceptor proteins as phosphotyrosine and histone (Ågren and Ronquist 1974, Ronquist and Ågren 1974 b, Mastro and Rozengurt 1976). Ehrlich cells prelabeled *in vivo* with (32 P) orthophosphate either alone or in combination with (14 C) glucose are incubated with human glioma cells in culture resulting in the recovery of labeled phosphorylserine and phosphorylthreonine from the glioma cells. Some of the results have been published in a preliminary note (Ågren and Ronquist 1976 b). The results support a proposed model for possible interaction between cells (Ronquist and Ågren 1975). The model requires in addition ATP at the cell surface. An ATP formation at the cell surface has been shown to occur in both Ehrlich and glioma cells (Ågren and Ronquist 1969 Ågren *et al.* 1971).

Material and methods

Media. All chemicals are of analytical grade. (γ - 32 P) ATP (32 P) orthophosphate D-glucose [14 C(U)] and L-leucine [14 C(U)] are purchased from NEN Chemicals, GmbH, Frankfurt am Main, W. Germany. Labeled ATP (sodium form), cyclic 3',5' AMP and ADP are obtained from Regma Chemical Company, Los Angeles, USA.

Preparation of Ehrlich cells. The Ehrlich ascites tumor cells were grown for 8-10 days in 5-weeks old mice. Sera albumin was obtained from the Anckerström breeding farm, Norrby, Stockholm. The Ehrlich cells were prelabeled *in vivo* with 1 mCi of (32 P) orthophosphate either alone or in combination with 100 μ Ci of (14 C) glucose. In the double-labeled experiments 100 μ Ci of (14 C) glucose had also been injected intraperitoneally 24 h prior to sacrifice. In one series of experiments Ehrlich cells were prelabeled *in vivo* with 10-20 μ Ci of (14 C) leucine 24 h before incubation. Ehrlich cells were separated by centrifugation of ascitic fluid. Each had been washed three times with ice-cold Krebs-Ringer bicarbonate medium, in order to eliminate the activity of cell adhesion. The cells were then washed twice in the Krebs-Ringer bicarbonate medium. Final washing is performed with medium of 130 mM NaCl and 25 mM KCl just prior to incubation.

Preparation of plasma membrane fractions from prelabeled Ehrlich cells

Preparation of plasma membrane fractions from prelabeled Ehrlich cells was in accordance with the procedure given in previous work (Wernstedt *et al.* 1975) with the exception that the EGTA-treatment during preparation is excluded. This is necessary since the observation is made that complete lysis of the microsomal fraction occurred when the (32 P) orthophosphate injections had been given to the mice. This phenomenon is also seen when the radioactivity of (32 P) orthophosphate is increased to 81 mCi per animal.

Tissue culture conditions and preparation of glioma and glioma cells for incubation experiments

The cells were grown in Eagle minimum essential medium with 10% calf serum supplemented with penicillin, streptomycin and fungizone. All cultures are grown at 37°C in 5% CO₂-humidified air. After twofold washing at 37°C in the isotonic saline solution the cells were immediately used for incubation experiments.

Incubation procedures

L. Experiments (γ - 32 P) ATP added to glioma and glioma cells. The washed glioma or glioma cells were incubated at 37°C with 10 mmoles of (γ - 32 P) ATP to each plate (totally 40 plates are used) in an isotonic medium containing 130 mM NaCl and 25 mM KCl, 10 mM TrisHCl-buffer pH 7.5, 3.5 mM MgCl₂, 0.7 mM reduced form of glutathione, 0.7 mM glutathione, reduced form (ordinary medium). In some experiments (Table 1) 75 mM

Phosphoryl group transfer from Ehrlich cells prelabeled *in vivo* with (^{32}P)-orthophosphate and (^{14}C)-glucose to unlabeled glioma cells

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Abstract

ÅGREN G and G RONQVIST. Phosphoryl group transfer from Ehrlich cells prelabeled *in vivo* with (^{32}P)-orthophosphate and (^{14}C)-glucose to unlabeled glioma cells. *Acta physiol. scand* 1978 103: 320-330.

A transfer of (^{32}P)-labeled phosphoryl groups has been demonstrated to occur between prelabeled Ehrlich cells (donor cells) and glioma cells (acceptor cells). The Ehrlich cells were generally prelabeled *in vivo* by an intraperitoneal injection of (^{32}P)-orthophosphate 4 hours prior to sacrifice of the donor bearing mice. In some experiments two injections of (^{14}C)-glucose were given to the animals in addition to the injection of (^{32}P)-orthophosphate prior to sacrifice. The prelabeled Ehrlich cells were mixed with the glioma cells, that were still attached to the culture plates, and incubation took place *in vitro*. Labeled (^{32}P)-phosphoryl (^{14}C)-serine was isolated from the glioma cells as well as from the Ehrlich cells. The $^{32}\text{P}/^{14}\text{C}$ ratio of (^{32}P)-phosphoryl (^{14}C)-serine of the glioma cells (acceptor cells) was significantly higher than for the Ehrlich cells (donor cells). This was indicative of interactive mechanisms between the cells and partly due to binding of membrane fragments from the Ehrlich cells on to the surface of the glioma cells and partly due to phosphoryl group transfer between the two types of cells. Interaction experiments with a plasma membrane fraction prepared from the prelabeled Ehrlich cells as well as the glioma cells clearly showed that the phosphoryl group transfer mechanism between the cells was dominated by only a minor part of the interaction was due to adhesion. This transphosphorylation reaction represents a new type of interaction between cells. It was not influenced by either adenosine 3,5'-monophosphate or adenosine 5'-diphosphate.

A membrane bound protein kinase which phosphorylates endogenous membrane proteins was first found in Ehrlich ascites tumor cells (Ågren and Ronqvist 1970, Ronqvist and Ågren 1970, Ågren and Ronqvist 1971). Using exogenous ($\gamma\text{-}^{32}\text{P}$)ATP as substrate, it was apparent that the reaction took place at the outer surface of the intact Ehrlich cells as a result of phosphorylation of protein-bound seryl and threonyl-residues of the acceptor protein(s) (Ågren and Ronqvist 1970, Ronqvist and Ågren 1970, Ågren and Ronqvist 1971, Ronqvist and Ågren 1974 a). Protein kinase-catalyzed membrane phosphorylation also involving the endogenous protein kinase and extrinsic acceptor protein or an extrinsic enzyme and the endogenous acceptor or where both acceptor and enzyme are endogenous

TABLE I (37) phosphoryl group transfer from (γ - 32 P) ATP into cell surface of gila and glioma cells. A single value from 2 expts are given in picomoles per $1 \cdot 10^6$ cells.

	Gila cells	Glioma cells
phosphorylserine residues	1.49	0.33
phosphorylthreonine residues	0.37	0.05

exogenous phosphoryl group acceptor protein(s) seemed to be less labeled in glioma cells than in gila. This is congruous with the earlier findings of less protein kinase activity at the cell surface compared with gila in the presence of exogenous acceptor protein (Zan and Ronquist 1974). On the contrary Rozengurt and Heppel (1975) compared the normal and virus-transformed malignant pair of Swiss 3T3 cells and claimed that the normal cells were phosphorylated by exogenous ATP while the normal cells were not. Fig. 1 A-E illustrate the Ehrlich cell behaviour on the plates during the incubation and washing procedures. Fig. 1 A illustrates washed glioma cells attached to the plates immediately before incubation. Fig. 1 B shows Ehrlich cells and glioma cells during incubation. It is evident that the Ehrlich cells cover the glioma cells completely. Fig. 1 C shows that Ehrlich cells are still present after the first washing. The number of Ehrlich cells is even reduced after the second washing (Fig. 1 D) while no Ehrlich cells are visible after the third washing (Fig. 1 E).

As is given in Table II it was possible to isolate measurable amounts of labeled phosphorylserine and phosphorylthreonine in four consecutive experiments on different samples from the glioma cells after incubation with Ehrlich cells prelabeled *in vivo* with (γ - 32 P) orthophosphate. There might exist different modes for the quantitative determination of phosphoryl groups transferred from donor to acceptor cells. One is based on the assumption that about 1% of the ATP content of the Ehrlich cells is available at the cell surface (Ägren and Ronquist 1969; Wernstedt *et al.* 1975) and that the (γ - 32 P) orthophosphate has reached equilibrium with the different phosphate pools of the Ehrlich cell. On this basis the mean values for phosphoryl group transfer from Ehrlich to glioma cells were 0.37 picomoles of labeled phosphorylserine per $1 \cdot 10^6$ glioma cells. The corresponding figure for phosphorylthreonine was 0.060 picomoles (Table II). Correction has not been made for destroyed phosphorylserine and phosphorylthreonine during acid hydrolysis. It is also seen in Table II that ADP or cyclic AMP did not influence the transphosphorylation reaction. Also, cyclic AMP has not shown any stimulating activity on the membrane protein kinase of Ehrlich cells and glioma cells in earlier experiments with exogenous (γ - 32 P) ATP as the phosphoryl group donor and endogenous acceptors (Ronquist and Ägren 1974; Ägren and Ronquist 1974; Ronquist *et al.* 1977). A modified Eagle's medium (Ca²⁺-free) was used in these experiments instead of the buffered saline solution. The labeled phosphoryl group transfer into Ser- 32 P under otherwise identical conditions was reduced by about 50%. One possible explanation for that might be that the (γ - 32 P)ATP at the cell surface of the donor cells is less available for the transphosphorylation reaction in the presence of albumin in the incubation medium (cf. Fig. 3 in Wernstedt *et al.* 1975).

In an experimental system comprising phosphoryl group transfer between cells it is

II) ADP and 3,5 AMP were included respectively. Furthermore, in another experiment the isotonic and K⁺ solution buffered with TrisHCl was changed for Eagle's medium lacking Ca²⁺. Total incubation volume was 3 ml. Incubation proceeded for 1 min and was terminated by pouring off the incubation medium from the plates containing the attached cells. The cells were further washed three times in the new NaCl-KCl-medium prior to precipitation with 3 ml of warm 3 per cent sulfosalicylic acid (SSA) and in the acid medium for 3 min. The SSA-treatment was repeated once. The destroyed cells were then wet in a minimal amount of SSA until removal of the cells from the plate (Rönquist *et al.* 1974).

2. (³²P) orthophosphate pre-labeled Ehrlich cells incubated with glioma cells. After the final washing, pre-labeled Ehrlich cells were immediately suspended in the above-mentioned incubation medium and (³²P) ATP. The cytocrit was adjusted to give a total of about 12 million cells per 3 ml cellsuspension. The suspension was kept in ice-water and under oxygen (96%) carbon dioxide (4%) atmosphere until use. Each plate of the glioma cells (about 6 millions per plate) were incubated after the final washing with 3 ml of the Ehrlich cell suspension pretreated in a water bath at 37°C immediately before the incubation experiment. Incubation proceeded for 3 min and was terminated by pouring off the Ehrlich cells. The plate still containing the attached glioma cells. The incubated glioma cells were washed and treated with SSA as described above.

3. ¹⁴C-glucose and (³²P) orthophosphate double-labeled Ehrlich cells incubated with glioma cells. Double-labeled Ehrlich cells were incubated with glioma cells exactly as described above. The treatment of the incubated glioma cells was also in accordance with the above-mentioned description. The double-labeled Ehrlich cells were also washed three times in the isotonic saline solution after incubation. The cells were then precipitated in 5 per cent trichloroacetic acid (TCA). The desaturated Ehrlich cells were washed once in 5 per cent TCA containing an excess of unlabeled orthophosphate and ATP.

4. C-leucine-prelabeled Ehrlich cells incubated with glioma cells. In order to rule out possibilities of labeled membrane fragments from the pre-labeled Ehrlich cells on to the glioma cell surface, the following controls were carried out. The Ehrlich cells were pre-labeled *in vivo* with 10–20 µCi of leucine 24 h before the incubation with the unlabeled glioma cells. The incubation and washing procedure were identical with those described above with one exception. After the incubation the glioma cells were washed three times with the isotonic salt solution followed by three washings with hot (90°C) SSA. As is seen in Table III the first and second washings from the incubated glioma cells contained radioactive material contrary to the third washing.

5. Plasma membrane fraction prepared from (³²P) pre-labeled Ehrlich cells incubated with glioma cells. A plasma membrane fraction was prepared from 3.6 · 10⁶ Ehrlich cells pre-labeled *in vivo* with (³²P) orthophosphate by lysis of 0.1 M (³²P) orthophosphate in each a total. The labeled membrane fraction corresponded to about 45 mg protein. The membrane material was prepared just prior to incubation and maintained at 0°C until incubation. The material was equally distributed 1 to 39 plates containing washed glioma cells. Incubation proceeded for 3 min at 37°C and was terminated by pouring off the labeled plasma membrane fraction. Washings of the incubated glioma cells were in accordance with the procedure given above and the glioma cells and membrane material from the Ehrlich cells were treated with hot 3% SSA as given above.

Acid hydrolysis of cell material and isolation of labeled phosphorylserine and phosphorylthreonine

After incubation the acid-treated Ehrlich cells and plasma membrane fraction were concentrated according to Schneider (1945) and partially hydrolyzed following the method of Lipmann (1933). The small amount of glioma cell material did not permit Schneider procedure and was therefore directly hydrolyzed as given above. Labeled phosphorylserine and phosphorylthreonine were isolated by Dowex 50 column chromatography as previously described (Ågren and Rönquist 1970, Rönquist and Ågren 1970).

Radioactivity was determined in a Nuclear Chicago Scintillation Counter and ³²P radioactivity determined by measuring the Cherenkov radiation. In the experiments with double-isotope labeling, ³²P-determination was performed in connection with the experiment while the ¹⁴C-determination was made after about 2 months in order to facilitate the discriminative measurement.

Cell counts were done electronically. Linscon counter 411 celloscope.

Results

Table I demonstrates the presence of endogenous acceptor proteins at the cell surface of both glioma and glioma cells using (³²P) ATP as exogenous phosphoryl group donor. The

(³²P) phosphoryl group transfer from donor cells into cell surface of acceptor cells under aerobic conditions. The values are given in picomoles per $1 \cdot 10^6$ cells. ADP concentration was 0.73 mM and 3',5'-cyclic AMP concentration was $1 \cdot 10^{-4}$ M. Calcium ions were excluded from the Eagle's medium. Range of values for phosphorylserine and phosphorylthreonine residues from 4 experiments are given in brackets.

	Ordinary medium	ADP in medium ^a	3',5'-cyclic AMP in medium ^b	Eagle modified medium ^c
phosphorylserine residues	0.37 (0.25-0.54)	0.33	0.32	0.16
phosphorylthreonine residues	0.06 (0.02-0.11)	0.07	0.06	—

^a 10% v/v
^b 10% v/v
^c 10% v/v

to exclude the possibility of transfer of peptides and membrane fragments. In fact, the occurrence of peptides and proteins at the outer surface has been reported (Gates *et al.* 1974, Rozengurt and Heppel 1975). Therefore, experiments were made with Ehrlich cells prelabeled *in vivo* with ¹⁴C-leucine. Such cells were incubated with glioma cells (Table III). The ¹⁴C-leucine content of Ehrlich cells after incubation with the glioma cells (3 washings with isotonic salt solution are included) was 122 512 cpm. In the Table, the "blind" washings (empty plates incubated with Ehrlich cells with ¹⁴C-leucine) with hot 3% SSA did not contain any radioactivity while remaining washed and SSA-treated glioma cells contained 64 cpm, i.e. 0.04 per cent of the total activity of the Ehrlich cells. Since the detached, incubated glioma cells always showed a small ¹⁴C-activity the possibility of a transfer of labeled fragments from the Ehrlich cells could not be completely excluded. Hence Ehrlich cells were also labeled on two different occasions with (¹⁴C)-glucose as well as with (³²P)-orthophosphate. The double-labeled (¹⁴C)-serine (³²P)- and (¹⁴C)-threonine (³²P)-phosphoryl residues were isolated in two ways both from the donor cells (Ehrlich cells) and from the acceptor cells (glioma cells). The amount of labeled residues were calculated by measuring (¹⁴C)- and (³²P)-

Table III ¹⁴C-radioactivity of prelabeled Ehrlich cells, the three washings and glioma cells after incubation. The Ehrlich cells were prelabeled *in vivo* 24 h before incubation with the glioma cells with 10-20 μ Ci of ¹⁴C-leucine. The washed labeled Ehrlich cells are incubated with the glioma cells as given in text. The figures denote total counts per min.

Total radioactivity (cpm)

	Ehrlich cell suspension after incubation	1st washing with 3% SSA	2nd washing with 3% SSA	3rd washing with 3% SSA	Detached glioma cells after the washings
serine containing cells	122 512	4 524	1 416	0	64
threonine containing cells	130 304	1 416	0	0	0

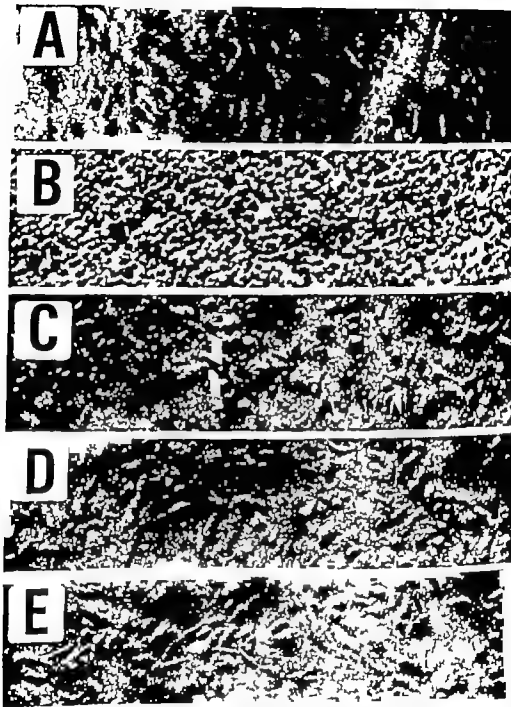


Fig. 1. A. Photograph of washed glioma cells attached to the plates immediately before incubation. B. Prelabeled Ehrlich cells ($1 \cdot 10^5$ cells) cover completely the glioma cells ($1 \cdot 10^4$ cells). Photograph taken during incubation. C. Glioma cells after completion of incubation. The Ehrlich cells have been separated from the glioma cells and they have been washed once with the isotonic salt solution. D. Ehrlich cells are still seen among the glioma cells. E. Ehrlich cells are no longer visible after the third washing of glioma cells.

TABLE II (7) Phosphoryl group transfer from donor cells into cell surface of acceptor cells under various conditions. The values are given in picomoles per 10^6 cells. ADP concentration was 0.73 mM and 3',5'-cyclic AMP concentration was $1 \cdot 10^{-6}$ M. Calcium ions were excluded from the Eagle's medium. Range of values for phosphorylserine and phosphorylthreonine residues from 4 cells are given in brackets.

	Ordinary medium	ADP in medium ^a	3',5'-cyclic AMP in medium ^b	Eagle's modified medium ^c
Phosphorylserine residues	0.37 (0.25-0.54)	0.35	0.32	0.16
Phosphorylthreonine residues	0.06 (0.02-0.11)	0.07	0.06	—

^a High exp.
^b Same value of 2 exps.

In order to exclude the possibility of transfer of peptides and membrane fragments, i.e., the occurrence of peptides and proteins at the outer surface has been reported (e.g., Gates *et al.* 1974, Rozenbaum and Heppel 1975). Therefore, experiments were carried out with Ehrlich cells prelabeled *in vivo* with 14 C-leucine. Such cells were incubated with glioma cells (Table III). The 14 C-leucine content of Ehrlich cells after incubation with the glioma cells (3 washings with isotonic salt solution are included) was 122 512 cpm. As also seen in the Table, the "blind" washings (empty plates incubated with Ehrlich cells prelabeled with 14 C-leucine) with hot 3% SSA did not contain any radioactivity while the remaining washed and SSA-treated glioma cells contained 64 cpm, i.e. 0.04 per cent of the total activity of the Ehrlich cells. Since the detached, incubated glioma cells always retained a small 14 C-activity the possibility of a transfer of labeled fragments from the Ehrlich cells could not be completely excluded. Hence Ehrlich cells were also labeled on two different occasions with (14 C)-glucose as well as with (32 P)-orthophosphate. The double labeled (14 C)-serine (32 P)- and (14 C)-threonine (32 P)-phosphoryl residues were isolated in a similar way both from the donor cells (Ehrlich cells) and from the acceptor cells (glioma cells). The amount of labeled residues were calculated by measuring (14 C)- and (32 P)-

TABLE III 14 C-radioactivity of prelabeled Ehrlich cells, the three washings and glioma cells after incubation. The Ehrlich cells are prelabeled *in vivo* 24 h before incubation with the glioma cells with 10-20 μ Cl of 14 C-leucine. The washed labeled Ehrlich cells were incubated with the glioma cells as given in text. The figures denote total counts per min.

Ehrlich cell suspension after incubation	Total radioactivity (cpm)						Detached glioma cells after the washings
	1st washing sch 3		2nd washing sch 3		3rd washing sch 3		
	SSA	SSA	SSA	SSA	SSA	SSA	
TY containing donor cells	122 512	4 524	1 416	0	0	0	64
TY plates	198 304	1 416	0	0	0	0	0

TABLE IV Radiactivity of double labeled (^{32}P)-phosphoryl (^{14}C)-serine residues in donor cells and acceptor cells. Ehrlich cells were prelabeled *in vivo* twice 52 h and 24 h before incubation. At first and second prelabeling 100 μCi of ^{14}C -glucose were administered intraperitoneally. 1 μCi of (^{32}P)-orthophosphate was also included in the second injection. Figures denote the specific (^{32}P)-phosphoryl and ^{14}C -radioactivity in counts per min per $1 \cdot 10^6$ donor and acceptor cells respectively

	(^{32}P) phosphoryl radioactivity	^{14}C radio- activity	$^{32}\text{P}/^{14}\text{C}$ ratio
Donor cells (Ehrlich cells)	181 083	18 973	9.6
Acceptor cells (glioma cells)	1 129	67	16.9

radioactivity. The results are given in Table IV. It is seen that labeled phosphorylserine residues were recovered from the glioma cells amounting to 0.62% of the total Ser ^{32}P of the donor cells calculated on P measurements. If instead this measurement of the recovered labeled phosphorylserine residues was made on ^{14}C -counting of phosphoryl-(^{14}C) serine the corresponding figure amounted to only 0.35%. Thus, there is a discrepancy between the (^{32}P)-phosphoryl and ^{14}C -countings of the recovered phosphorylserine residues of glioma cells indicating a changed stoichiometric relationship between the (^{32}P)-phosphoryl-(^{14}C) serine residues of the donor cells and (^{32}P)-phosphoryl (^{14}C)-serine residues of the acceptor cells favouring the view that the increased $^{32}\text{P}/^{14}\text{C}$ ratio of the serine residue of the acceptor cells is due to a transphosphorylation from the donor cells into the acceptor cells.

Table V illustrates that 4.1 per cent of the total (^{32}P)-radioactivity of the Ehrlich cells are recovered in the plasma membrane fraction. Furthermore, 0.11 per cent of the total activity are recovered in the (^{32}P)-phosphoryl residues after partial hydrolysis of this plasma membrane fraction. It is also seen in the table that the total cell count corresponds to 1.05 mg protein. 4.3 per cent of this amount is recovered in the plasma membrane fraction. This figure is close to the percentage value of radioactivity distribution. It is noteworthy since the radioactivity and protein measurements were made on different cell preparations. The set of 6 values given in Table VI has been obtained on 6 different expts. performed during a two years period. A mean value of 0.019 per cent of the total (^{32}P)-radioactivity of the Ehrlich cells is recovered in the (^{32}P)-phosphorylserine residues of the glioma cells.

TABLE V Distribution of (^{32}P)-radioactivity and protein in Ehrlich cells and subfractions from the cells. The Ehrlich cells were prelabeled *in vivo* as given in text

	(^{32}P) radioactivity in cpm. (millions)	Per cent of total radioactivity	Protein con- tent (mg)
Ehrlich cells ($3.6 \cdot 10^6$)	171.1	100	1.050
Plasma membrane fraction	7.1	4.1	0.45
Plasma membrane fraction purified according to Schneider	2.7	1.6	
(^{32}P)-phosphorylserine residues isolated after partial hydrolysis	0.18	0.11	

TABLE VI Relationship between total counts in Ehrlich cells and (32 P)-phosphorylserine residues of glioma cells

Total counts in Ehrlich cells (cpm in maltese)	Total counts of (32 P)-phosphorylserine residues of glioma cells (cpm)	Per cent recovery of radioactivity in (32 P)-phosphorylserine residues of glioma cells
111	756	0.011
36	1364	0.025
163	2238	0.014
160	3870	0.024
95	2997	0.032
145	1463	0.010

Mean per cent value from 6 different expts. = 0.019

It is known that cell membrane fragments can bind to the surface of intact cells under certain conditions (Öbrink *et al.* 1977). Therefore it was necessary to exclude the possibility of a binding between membrane fragments from the Ehrlich cells and the surface of the glioma cells. A plasma membrane fraction was prepared from 3.6×10^6 Ehrlich cells labeled *in vivo* with (32 P)-orthophosphate. The radioactive membrane fraction, corresponding to 45 mg protein, was incubated with the glioma cells. An extremely small amount of (32 P)-phosphorylserine residues barely detectable with radiometric methods was recovered from the incubated glioma cells (Table VII). The figure obtained from the glioma cells only amounted to 1.9 per cent of the expected value calculated on an ordinary interaction between the prelabeled Ehrlich cells and the glioma cells (see Tables V and VI).

Discussion

The interaction between two types of cells as demonstrated in the present paper requires basically some prerequisites. They include the presence of protein kinase and an endogenous phosphoryl group acceptor at the cell surface. Furthermore, ATP is necessary as the

TABLE VII Radioactive Recovery of (32 P)-phosphorylserine residues from glioma cells after incubation with radioactive plasma membrane fraction from prelabeled Ehrlich cells. Relationship to expected (calculated) value for total radioactivity of (32 P)-phosphorylserine residues from glioma cells.

	Radioactivity in counts per min
Total radioactivity of plasma membrane fraction prepared from 3.6×10^6 Ehrlich cells	933 296
Total (32 P)-radioactive recovery of phosphorylserine residues from glioma cells	81
Expected (calculated) value for total radioactivity of (32 P)-phosphorylserine residues from glioma cells	4 318 ^a

^a Figure obtained by dividing 933 296 with factor 216.15

^b Factor obtained by multiplying 37.27 (4.1 \times 0.11 see Table V) with 5.8 (0.11 (Table V) \times 0.019 (Table VI)).

phosphoryl group donor. The presence of protein kinase at the cell surface has been demonstrated on different cells in our laboratory and confirmed by others (Mastro and Rozengurt 1976, Schlaeger and Köhler 1976). The endogenous acceptor is present at the cell surface of gila as well as glioma cells (Table I) although the latter cells display a low degree of phosphorylation compared with the former cells contrary to findings on each pair of normal and malignant cells (Rozengurt and Heppel 1975). The existence of a ATP forming capacity at the surface of Ehrlich cells has been discussed (Ågren and Ronquist 1969, Ågren and Ronquist 1975, Wernstedt *et al.* 1975). However one might argue that (32 P)-orthophosphate may permeate from the donor cells into the medium and the medium into the acceptor cells. Labeled orthophosphate can then be incorporated in the terminal position of ATP by glycolysis in the latter cells and thereafter take part in phosphorylation reactions of these cells. In order to nullify the phosphate gradient into the incubation medium all experiments were carried out in the presence of $1 \cdot 10^{-4}$ M unlabeled orthophosphate. Furthermore, even in the absence of equilibrated gradients, the phosphate transport through the plasma membrane is slow (Ågren and Ronquist 1969). Moreover when exchanging the exogenous (32 P) ATP for (32 P) orthophosphate in the incubator medium, the labeling of the endogenous peptide of the surface membrane of Ehrlich cells was negligible even after a 5 min incubation period (Ågren and Ronquist 1971). Therefore it seems unlikely that the phosphoryl group transfer studied was caused by labeled orthophosphate transported from the Ehrlich cells into the glioma cells.

Another possibility would be the binding of possible labeled membrane fragments from the Ehrlich cells on to the surface of glioma cells. Such an adhesive specificity between some other cell types has been claimed to occur (Öbrink *et al.* 1977). However this binding was confined to one cell type and certain organelles from these cells. Experiments performed with 14 C-leucine-prelabeled Ehrlich cells could favour such a hypothesis since a slight amount of 14 C-labeling was recovered in the glioma cells after incubation with the Ehrlich cells (Table III). Experiments with Ehrlich cells, double labeled with 14 C-glucose and (32 P)-orthophosphate again seemed to indicate a certain small degree of binding, since the recovered (32 P)-phosphorylserine residue fraction not only contained (32 P)-radioactivity but also some 14 C-radioactivity (Table IV). The 32 P/ 14 C ratio was however different for the acceptor cells compared with the donor cells suggesting in addition a phosphoryl group transfer. It should however be kept in mind that the phosphorylserine residue fraction is heterogeneous (Ågren *et al.* 1962, Ronquist and Ågren 1974 a) containing several non-essential amino acids as well as hexosamine. These compounds might contain a high-specific labeling of 14 C-carbon while lacking (32 P)-labeling. Thus, a minor component of binding of membrane fragments from the Ehrlich cells onto the surface of glioma cells cannot be ruled out. A comparison is difficult to perform stoichiometrically since one molecule of e.g. hexosamine can be labeled maximally on all six carbons atoms while a molecule of phosphorylserine can only maximally be 32 P labeled in one position. Hence, it was not possible to estimate on a quantitative basis how much of the recovered labeling in the glioma cells was due to phosphoryl group transfer and binding of membrane fragments, respectively.

A promising step forward along this line seemed to be the incubation experiments with

the plasma membrane fraction prepared from prelabeled Ehrlich cells. Such a plasma membrane preparation contained about 4 per cent of the total (32 P)-radioactivity of the Ehrlich cells. The preparation of the plasma membrane fraction included several purification steps and it seemed likely that loosely bound low molecular compounds and cofactors including ATP had been removed. Therefore, since apparently no phosphoryl group transfer was possible, conditions were available for study of a possible binding reaction between the plasma membrane fraction containing (32 P)-phosphorylserine and glioma cells. It was clearly demonstrated that only 2% of the (32 P)-labelling of the glioma cells could be due to loading of membrane fragments onto the surface of glioma cells while the majority of the labelling most probably was caused by a phosphoryl group transfer.

In conclusion we have demonstrated a new type of interaction between cells involving a phosphoryl group transfer. The physiological significance of such a reaction is at present not clear but one possibility might be a regulatory phosphorylation at the cell surface (cf ref. Jonquist *et al* 1977). This also implies a dephosphorylation reaction at the cell surface. Such a reaction has recently been found at the surface of intact Ehrlich cells as well as in the plasma membrane fraction (unpublished results).

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Fractional recovery following cross-reinnervation of antagonistic forelimb muscles in rats

By

AVIS H. COHEN

Several early studies in mammals (Barron 1934 and Sperry 1947) indicated the return of normal function following cross-innervation of antagonistic muscles. Mammalian muscle is generally believed to be innervated by only one nerve fiber. If a second axon does innervate a muscle fiber both sets of neuromuscular junctions are functional (Frank *et al.* 1975). Thus, it is suggested that once a muscle is cross-reinnervated normal function can only be restored by neural reorganization. The results of Sperry (1942, 1947) denied that such reorganization could occur. The present study in rats reexamines this problem.

Single pairs of antagonistic forelimb muscles were cross-innervated and their subsequent electromyographic (EMG) pattern examined. Unlike Sperry's rats the animals were left otherwise intact. Sperry had removed the remaining limb musculature, the paw, the contralateral limb and the tail. Although the majority of animals in the present study confirmed Sperry's findings, some did display alteration in the activity of the crossed muscle nerve.

Twelve 40-60 g weanling albino rats were anesthetized with sodium pentobarbital (50 mg/kg) and the nerves to the medial head of the triceps (MhT), an extensor and the long head of the biceps (LhB), a flexor were dissected to the point of entry into the muscles. A very fine silicon monolaminate wire (Ethicon, 8-0) was laid across the nerve and small pieces of underlying muscle tissue, and reattached to the antagonistic muscle at the myotome level. Once the original nerve had been removed, sham operated animals ($n = 2$) were tested at the same day but the nerves were reattached to their original muscles.

After 4-6 weeks electromyography was used during locomotion to test the function of the cross-innervated MhT muscles. The long head of the triceps was the control for the crossed MhT. During each trial, these two heads of the triceps fire at the same time. All tests included bipolar EMG recordings from the ventral long head of the triceps (LhT) and operated medial head of the triceps (MhT) (see Cohen and Chase 1977 for technique). Consistent bursts signified the MhT was functioning normally. Animals were run at least twice with separate electrode insertions.

In 10 animals innervation to the reinnervated MhT's was evaluated by determining the twitch tension produced by stimulating the flexor and extensor nerves, including 2 whose MhT's had exhibited only sensory-type activity (see below). Under anesthesia anesthesia (1.5 g/kg) all the forelimb musculature was removed except the MhT and LhT. The proximal extremities of the flexor and extensor nerves were exposed and cut. The humerus was fixed. The MhT was placed at an anatomically appropriate angle, with its tendon attached to a monometric strain gauge and the muscle cut to produce maximal twitch tension. The nerves were mounted on bipolar stimulating electrodes and the muscles bathed in paraffin oil (38°-39°).

Tension measurements revealed that in the MhT's of 7 animals tested there was flexor and extensor innervation. In 3 animals only the foreign flexor innervation produced tension (Table 1, see Legend).

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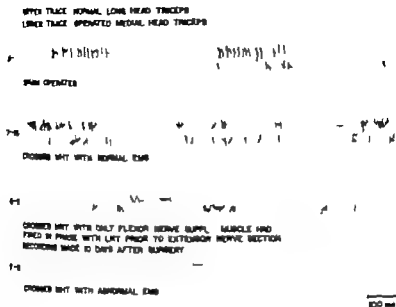


Fig. 1. EMO recordings of 4 animals. The upper trace of each pair is the normal long head of the triceps and the lower trace is the operated medial head of the triceps. The number above each pair of traces refers to the animal. Note, in animal 7-2, although there is no measurable tension produced by the triceps (Table I) there was recordable extensor-type EMG-activity. Thus, in view of the large tensions generated in theiceps nerve in MHT of 7-16 it is unlikely that the nerve is producing undetected flexor-type activity.

changes may be due to a modification in the "central program for locomotion" or to the establishment of a new program. It might, however, simply reflect a tonic suppression of the floor extensors centrally or by peripheral feedback from normal or abnormally regenerated sensory receptors. If further experiments can corroborate these results the view that no central reorganization can occur must be abandoned.

This work formed part of a doctoral dissertation submitted to the Division of Biological Sciences, Cornell University, and was completed under the direction of Drs R. R. Capra and Carl Gera. The mechanical properties of the crossed muscles were investigated in the laboratory of V. R. Edgerton, U.C.L.A., with the aid of Dr Edgerton, R. R. Ruppel and D. Simpson. Comments by Dr S. Grillner are extremely helpful in the production of this manuscript.

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TABLE 1 Twitch Tensions of the operated MhT's generated by stimulation of the foreign or original nerve. *f/e* = flexor/extensor-type EMG *e* = extensor-type EMG. There are 3 possible explanations for the extensor bursts in 6-6, 7-3 and 7-8: 1) The force transducer could not measure tension less than 1 g. Thus, if less than 1 g of tension was produced by extensor innervation, it could be undetectable. 2) the extensor nerve was injured during the dissection. 3) the flexor innervation was active during the extensor phase of the step cycle.

Animal	Condition EMG pattern	Tension (g) Nerve stimulated	
		Triceps	Biceps
6-3	crossed <i>f/e</i>	4.9	8.9
6-6	crossed <i>f/e</i>	0	4.3
6-13	crossed <i>f/e</i>	7.6	6.0
7-3	crossed <i>f/e</i>	0	9.3
7-5	crossed <i>f/e</i>	13.9	15.5
7-8	crossed <i>f/e</i>	0	15.9
7-9	crossed <i>f/e</i>	12.2	6.4
7-11	crossed <i>f/e</i>	11.0	3.1
6-9	crossed <i>e</i>	10.7	3.5
7-16	crossed <i>e</i>	10.7	12.0
7-17	sham	15.3	—

Flexor/extensor type EMG In 15 of 19 animals the EMG pattern consisted of two bursts of activity per step cycle: one in-phase with the control LhT (extensor-type burst), and one out-of-phase with the LhT (flexor-type burst). Presumably these bursts were produced by the extensor and flexor nerves, as the timing of these bursts was essentially identical to that seen in normal animals (Cohen and Gars 1975). The 15 animals included 3 with no measurable tension associated with the extensor nerve (cf. 7-8 Fig. 1 Table I).

Extensor type EMG In the remaining 4 animals the cross-reinnervated MhT's exhibited only one burst per stride (Fig. 1 7-16), coinciding with the activity of the LhT. The muscle was silent during the flexor phase of the step cycle. The presence of functional flexor innervation to the MhT's of two of these 4 animals was confirmed by stimulating flexor and extensor nerves. Both produced substantial tensions (Table I). With the techniques used it was impossible to determine whether the flexor nerve was contributing to the EMG activity.

Subsequently in a third animal from this group, under anaesthesia the extensor innervation to the MhT was cut as far proximal to the site of crossing as possible. This left only the muscle's flexor innervation intact. 5 days later the activity of the MhT was reexamined. During this and another test 10 days later the MhT exhibited only a strong flexor burst (Fig. 1 6-11). Six months later the activity was still pure flexor-type. The flexor EMG activity could have been a product of newly sprouted flexor nerve fibers, but it is unlikely that this would be a major factor just 5 days after nerve section. This result suggests that the suppression of flexor activity had not been a result of permanent rewiring.

In summary after the cross-reinnervation of antagonistic forelimb muscles, in 15 of 19 animals there were two bursts of EMG activity per step cycle. The periods of activity were those normally associated with the foreign flexor nerve and the original extensor nerve. Indeed the MhT's of most animals were found to be reinnervated by both nerves. However the MhT's of the 4 remaining animals exhibited only an extensor burst during locomotion, although their muscles were also found to have been reinnervated by both nerves.

This small group of animals is, nonetheless of considerable interest, as it indicates that changes in motor output can occur even in such a basic behavior as locomotion. These

UPPER TRACE: NORMAL LONG HEAD TRICEPS
LOWER TRACE: OPERATED MEDIAL HEAD TRICEPS

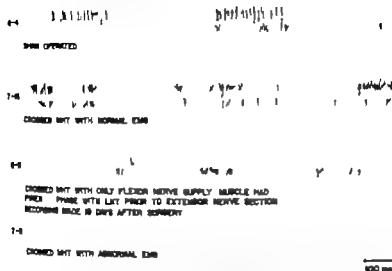


Fig. 1. EMG recordings of 4 animals. The upper trace of each pair is the normal long head of the triceps and the lower trace is the operated medial head of the triceps. The number above each pair of traces refers to the animal. Note, in animal 7-8, although there is no measurable tension produced by the triceps (Table 7) there is recordable extensor-type EMG-activity. Thus, in view of the large tension generated by the triceps nerve in NMT of 7-16 it is unlikely that the nerve is producing undirected flexor-type activity.

changes may be due to a modification in the central program for locomotion or to the establishment of a new program. It might, however, simply reflect a tonic suppression of the flexor motoneurons centrally or by peripheral feedback from normal or abnormally innervated sensory receptors. If further experiments can corroborate these results the view that no central reorganization can occur must be abandoned.

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Influence of dehydration on the phasic gustatory response to H_2O , D_2O , and $NaCl$ in the frog (*Rana temporaria*)

By

B. APPELOREN and S. ERIKSSON

Nearly 30 years ago Zotterman (1949) discovered that water applied to the frog's tongue produces a massive volley of large fibre impulses in the glossopharyngeal nerve. His subsequent comparative studies demonstrated the presence of water taste also in several mammalian species (cf. Zotterman 1956). Taste cells responding rather specifically to water have later been found also in certain insects (cf. Dethier 1977). This wide-spread species distribution suggests that water taste might fulfil some purpose in the homeostatic control of fluid balance. Therefore it was found of interest to study whether dehydration induced by exposure to a salty environment might change the gustatory response to water and $NaCl$ in the frog. Slow infusion of deuterium (D_2O) into the cerebrospinal fluid of goats has been found to inhibit the activity of apparently Na^+ -sensitive cerebral receptors involved in the control of fluid balance (Leksell, Lishajko and Rundgren 1976). For that reason the gustatory response to D_2O was also studied here.

Adult summer frogs, *Rana temporaria*, ($n = 22$) were used for recording of the afferent impulse activity in the glossopharyngeal nerve. Prior to the expts. 12 of the frogs were placed in 1% $NaCl$ solution for about 16 h which caused 5 to 7% reduction of body weight. The other 10 frogs were maintained in tap water until they were used for expts. The surgical preparation and the recording technique were principally as described by Zotterman (1949). During the expts. an impulse counter (SE Time-counter SM 700 Mk2) was continuously registering the nerve impulse frequency every 4th s (duration of each registration 1 s). The 20°C test solutions and the Ringer solution were applied to the tongue for 20 s at a constant flow of 5 ml/min. If the application of fluid happened to be started or discontinued during one of the 1 s registrations, that value was not included when calculating the induced mean increase in frequency. Prior to each test the response to the 20 s application of Ringer was recorded, and the observed increase in impulse frequency was subtracted from that obtained during the subsequent test period. Hence, the effect of mechanical stimulation of the tongue has been eliminated in the results presented. After each test period the tongue was again rinsed with Ringer. The test solutions used were Distilled H_2O , D_2O (99.8%, "Sigma"), 1% (171 mM) and 2% (342 mM) $NaCl$ in either H_2O or D_2O . The composition of the Ringer solutions was 115 mM $NaCl$, 2.5 mM KCl , 1.8 mM $CaCl_2$, 2.15 mM NaH_2PO_4 ($2H_2O$), and 0.85 mM $NaH_2PO_4(H_2O)$. The results obtained were subjected to analysis of variance using BMD Comput. Progr. Univ. Calif. Berkeley 1973.

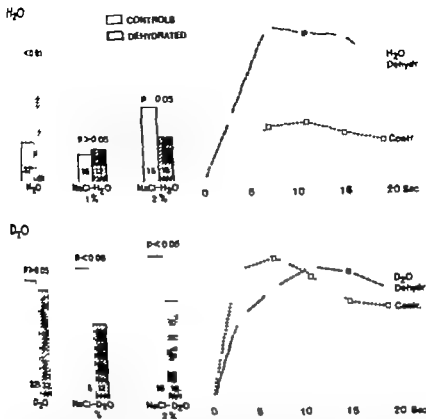


Fig. 1. Left: Mass increase or photoperforated isotope frequency in control ($n=10$) and dehydrated ($n=12$) frogs during the 20 sec application to the tongue of H_2O , D_2O and of NaCl dissolved in either H_2O or D_2O . To eliminate the influence of mechanical stimulation the mean isotope frequency elicited by repeated 20 sec applications of Ringer solution has been subtracted. Figures in the columns indicate porebers of test. Right above: Differences in time course and magnitude for the gustatory response to H_2O in dehydrated and control frogs. Right below: Lack of such differences for the response to D_2O .

As shown in Fig. 1 (left above) dehydration induced by exposure to a salty environment caused a conspicuous (200%) increase in the response to H_2O and reduced the effect of 2% NaCl/ H_2O by about 40%. No difference was obtained in the response to 1% NaCl/ H_2O between the dehydrated and control frogs. In the control frogs pure D_2O had a much stronger effect than H_2O (Fig. 1 left below). However this effect did not become accentuated after dehydration. Also the response to NaCl/ D_2O was considerably stronger than that obtained by NaCl dissolved in H_2O . The effects of both 1 and 2% NaCl/ D_2O were significantly greater in the control animals than in the dehydrated frogs.

The time courses of the responses to H_2O and D_2O in dehydrated and control animals are shown to the right in Fig. 1. In the control animals the reaction to D_2O was much faster than to H_2O . This was not the case in the dehydrated frogs, however.

Discussion. The fact that the gustatory response to H_2O became considerably accentuated after dehydration lends some support to the idea that the water taste may play a role in the

control of fluid balance of the frog. The manner in which the present frogs were dehydrated raises the question whether diminished amount of body water *per se* or elevated extracellular and/or intracellular $[Na^+]$ might have been the crucial cause of increased responsiveness to H_2O . No determinations of plasma or cellular $[Na^+]$ were made. However, the hypertonicity to H_2O was accompanied by a reduced gustatory response to 2% NaCl (Fig. 1 *below*). It indicates that elevated bodily $[Na^+]$ might have been the ultimate cause of the changed gustatory reaction in the dehydrated frogs.

The recorded effects of D_2O and NaCl/ D_2O were quite different from those obtained with H_2O and NaCl dissolved in H_2O . The present experiments do not allow any conclusions regarding the influence of D_2O on the taste receptors. However, the effect of D_2O appears to be rather nonspecific and not related to the "classical" water taste (Zotterman 1949). This is further indicated by the fact that no difference in the response to pure D_2O was observed between dehydrated and control frogs.

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Note on the conduction velocity of warm afferent fibres from the skin of the human leg

By

ULF NORRELL and MICHAEL ULLMAN

Measurements of reaction times to thermal stimuli have been used to investigate the neural basis of temperature sensitivity in man. The results from these experiments have sometimes generated conflicting theories (*cf.* Sinclair 1967) but the technique remains attractive since it offers the opportunity to correlate subjective experience with objectively quantifiable data. The results of recent reaction time experiments have indicated the subjective experience of "cold" and "warm" to depend on different populations of afferent neurones, and Fridericius (1976) has suggested a conduction velocity in the order of magnitude of 4 m/s for the "cold" afferents, and 2 m/s for the "warm" afferents. The present experiment was made in order to extend our knowledge with regard to the latter group.

We have measured the reaction times to radiation pulses which were applied to different places on one leg of 10 men and 10 women. The subjects were seated in a comfortable reclining chair. Their eyes and ears were occluded or plugged, which were located between the groin and the ankle and had been padded with cotton wool. They had been instructed to press a manual pushbutton as soon as they felt stimulation caused by the radiation pulse, which was generated by emitting the radiation from a 250 W Philips soldering lamp through a rotary shutter. The stimulation intensity was altered by changing the lamp voltage and the radiation was directed to strike the skin perpendicularly on a circular area of 40 mm diameter with an intensity gradient of 2/1 from the centre to the periphery. The reaction time was measured in seconds between the beginning of the radiation pulse and the pressing of the pushbutton.

Fig. 1 shows the reaction times obtained from the stimulation of a place located ventrally in the thigh 15 cm below the groin in one subject plotted against the stimulation intensity. The stimuli were applied at 60 s intervals and the figure shows that the reaction times were quite constant over a wide range of intensities but increased towards the lower end, close to the threshold for the responses. Stimuli of intensities below the threshold were introduced at random in all the testing sessions and served the double purpose of indicating the approximate sensitivity of the stimulated place as well as the specificity of the test, i.e. that reaction times to the radiation pulses were being measured and not those to parallel acoustical cues. The subjects were asked to describe their subjective experience of the stimulus after each trial and the descriptions fell into 3 categories, which have been indicated by different symbols in Fig. 1. The mean value and standard deviation of the reaction times to stimuli of the intensities which elicited purely warm sensations, i.e. those represented by circles in Fig. 1, have been plotted in Fig. 2D against the estimated distance from the dorsal roots together with the corresponding data from 3 more places on the same leg, which were in-

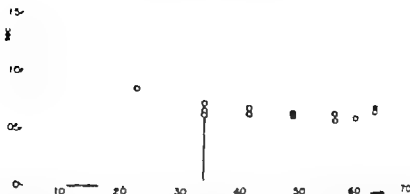


Fig. 1. Reaction times (ordinate) of manual pushbutton responses to 0.15 s radiation pulses which were applied to the right thigh of one person, plotted against the amount of energy of the individual arm (abscissa). The different stimulation intensities were used in random order. The stimuli sometimes failed to elicit responses, critical bars and otherwise elicited 3 different kinds of sensations: qualitatively ambiguous triangles, warmth, circles, warmth plus prickling, star. The energy values are based on radiation measurements.

investigated during the same session (*cf.* also legend Fig. 2). Data which were obtained in the same way from the 5 other experimental subjects are also presented in Fig. 2, and the figure shows that in all instances the reaction times regressed approximately linearly in relation to a diminished distance between the place of stimulation and the central nervous system.

The absolute magnitudes of reaction times to thermal stimuli are correlated to several factors (*cf.* Sinclair 1967), three of which could explain the observed regression: 1) the magnitude of the sensations caused by the radiation pulses; 2) the distance between the skin surface and the warm receptors; 3) the conduction velocity of the primary afferent nerve.

1. The reaction times to stimuli causing greater sensations are shorter than those caused by mild stimuli. In the present experiment, however, the stimulation intensities were filtered over the whole range between threshold and pain, and thus the shorter reaction times from the proximal sites can only be explained in this context by a greater density of innervation of these sites compared to the located more distally. Sensations of warmth are very dependent of both spatial and temporal summation (Marks, Stevens and Tepper 1976) however, and differential innervation of the different sites therefore would produce different response thresholds. No such variation between the thresholds of the different stimulation sites was observed.

2. The progress of thermal energy in the human skin has been estimated between 0.5–1 mm/s (Hick 1952). If the conduction time of the afferent fibres is disregarded temporarily, then the differences between the various reaction times of Fig. 2 could be explained by a delay caused by the warm receptors of the skin of the ankle being located 0.5–1 mm deeper than those of the thigh. Nothing is known about such a variation in depth of the warm receptors, but if existing it ought to be reflected by a corresponding variation of the thickness of the skin. In the human leg the dermis appears to be equally thick in places where the epidermis has been found to be 70 µm thicker on the ankle than on the thigh (Southwood 1955, Whetton and Eccall 1973). That difference is obviously too small to explain the variation of the reaction times shown in Fig. 2, which therefore could depend mainly on the delay caused by the conduction time of the afferent nerves.

3. If the sensations of warmth caused by the radiation pulses are transmitted via a homogeneous afferent nerve population, and if the two factors which were discussed above could be considered of minor importance, then it should be possible to extrapolate the conduction velocity of these afferent nerves from the present data. It was found to be in average 0.9 m/s (s.d. 0.2) for the subjects of Fig. 2 when calculated with linear regression (least squares method) on the basis of all the data which were used to construct that figure. This conduction velocity

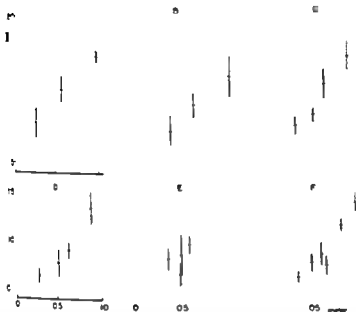


Fig. 2. Mean values, dots, and standard deviations, bars (N 8-21) of the reaction times to radiation pulses applied to 3-6 different places on the right legs of 6 persons. Each graph represents one subject and errors data collected during single sessions. Only the reaction time shows from stimulations which failed in unambiguous sensation of warmth have been used, and the first value of this group from each place of stimulation has been excluded. The different places were stimulated one at a time and the first place to be stimulated was always one on the middle of the leg. The distances to the different places of stimulation (submaximal) are measured from right angle projections on the longitudinal axis of the leg, which had been extended to an imagined zero located at the top of the sacral bone.

It is worth that of the few specific human warm afferent fibres which have been measured electrophysiologically (cf. Hensel 1976), and the present observations thus are taken to indicate that the sensations of warmth, which are elicited by radiation pulses in the human leg, are transmitted in unmyelinated primary afferents with conduction velocities of that order of magnitude.

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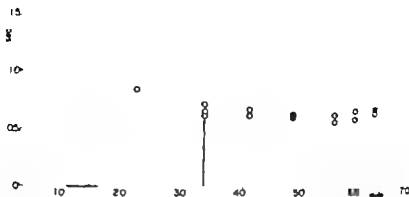


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1 The reaction times to stimuli causing greater sensations are shorter than those caused by weak stimuli. In the present experiments, however, the stimulation intensities were altered over the whole range between threshold and pain at each site and thus the shorter reaction times from the proximal sites could only be explained in this context by a greater density of innervation of these sites compared to those located more distally. Sensations of warmth are very dependent of both spatial and temporal resolution (Marks, Stevens and Teppe 1976), however, and a differential innervation of the different sites therefore would produce different response thresholds. No such variation between the thresholds of the different stimulation sites was observed.

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3 If the sensations of warmth caused by the radiation pulses are transmitted via a homogeneous afferent nerve population, and if the two factors which were discussed above can be considered of minor importance then it should be possible to extrapolate the conduction velocity of these afferent nerves from the present data. It was found to be in average 0.9 m/s (± 0.2) for the subjects of Fig. 2 when calculated with linear regression (least squares method) on the basis of all the data which were used to construct that figure. This conduction velocity

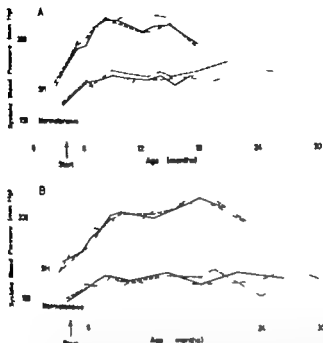


Fig. 1 Mean systolic blood pressure measured indirectly from the tail of spontaneously hypertensive (SH) and normotensive rats as a function of age: Control — 85 dB SPL noise - - - 105 dB SPL noise. Measurements are made before the animals were introduced into the boxes (at 4 months of age) and at one month interval during the first year thereafter and 3 months interval. Each value is based on ten daily measurements on each rat during 5 days. A: males, B: females.

is evident. Secondly all rats undergo a gradual rise of blood pressure except maybe the normotensive females. Thirdly the blood pressure after the start of the experiment does not broadly differ between exposed and non-exposed animals.

Averaged over all measurements taken each group during the first year after the start of the exposure, blood pressure for normotensive males was 130, 135, and 145 mmHg for environments A, B, and C respectively. The corresponding values for normotensive females were 130, 125, and 120 mmHg; for the spontaneously hypertensive males: 205, 210, and 215 mmHg; for spontaneously hypertensive females: 200, 205, and 208 mmHg.

There was no significant difference between any of the groups (one-way analysis of variance $p < 0.01$). Fourthly the spontaneously hypertensive males and females seem to undergo a more rapid initial increase in blood pressure in the 105 dB SPL noise than in the 85 dB or in control environments.

In order to clarify this latter point another 40 spontaneously hypertensive rats (20 in A and 20 in C) are later introduced into the experiment. Fig. 2 shows the average systolic blood pressure over the first 7 months for these two groups. There is no difference between exposed and non-exposed rats, either in males or in females.

The lack of effect of noise on blood pressure in this experiment may seem surprising in regard to the well-established short term effects and the earlier presented effects of intermittent noise over longer periods. Three possible explanations can be proposed. First, the

Noise and blood pressure. Effect of lifelong exposure in the rat

By

ERIK BORG and AAGE R. MÖLLER

Is noise a health hazard as far as non-auditory effects are concerned? Two contradictory opinions prevail. Kryter (1976) is very skeptical toward there being any harmful effect of noise outside those on the ear. Lehmann (1957) and Jansen (1973), on the other hand, go so far as to suggest criteria for noise with respect to deleterious effects mediated through the vegetative nervous system (especially those on the cardiovascular system). In the present work the hypothesis has been tested that meaningless noise can permanently influence cardiovascular homeostasis. The cardiovascular system has been chosen as a model since it is known that noise exposure of a short duration significantly alters e.g. blood pressure, heart rate, and peripheral vascular tone (see e.g. Hallböök and Folkow 1974, Borg 1977). Furthermore, disturbances in this system are likely to have generalized effects relevant to the concept of health.

In studies over a few months a rise of the blood pressure has been observed in rats during exposure to intermittent noise (Medoff and Bongiovanni 1945, Buckley and Smookler 1970). In those experiments sudden noise bursts were presented either during the daytime alone (sleep-time for rats) or both day and night. Acoustic stimulus having such a temporal pattern forms an unsatisfactory model for human noise exposure, especially with regard to industrial work. In this latter situation noise is more or less continuous during the active time of the day while sleep-time is relatively noise free.

The aim of the present investigation was to study the influence on blood pressure of lifelong exposure to a varied, meaningless noise presented during the greater part of the waking time of the rat (night).

A total of 130 rats of two strains were used in the present study: normotensive Sprague-Dawley rats and spontaneously hypertensive Wistar rats (Okamoto strain). From an age of 3-4 months they were kept in one of three sound-insulated boxes, each with one specific acoustic environment. Systolic blood pressure was determined by a "tail-cuff" technique (Borg and Jansson 1978) and heart girth was established after one year.

Environment A. Background noise produced by the rats themselves, approximately 50 dB (A).

Environment B. Noise exposure at 85 dB SPL.

Environment C. Noise exposure at 105 dB SPL.

The noise was presented through 4 Lansing L75 horns in each box. It consisted of a 1640 Hz broad noise band sweeping from 3 to 30 kHz at a rate of 0.5 Hz/s with a duty cycle of 50%, rise and decay time of interruption being 50 ms. The maximum sound energy in the range of 3-15 kHz was 85 and 105 dB SPL. Furthermore, the noise was randomly interrupted by a relay 7 times each night with a total pause time of 2 h.

The mean systolic blood pressure as a function of age is illustrated in Fig. 1 for exposed and non-exposed rats of both strains (A males, B females). 4 main observations can be made. First, the expected difference between normotensive and spontaneously hypertensive

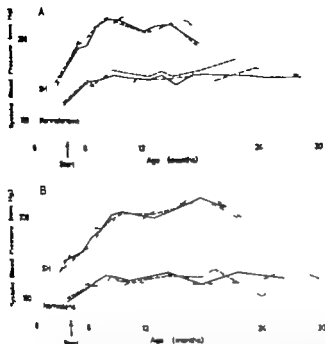


Fig. 1. Mean systolic blood pressure measured indirectly from the tail of spontaneously hypertensive (SH) and normotensive rats as a function of age. Control — 85 dB SPL noise - - - 105 dB SPL noise. Measurements are made before the animals were introduced into the boxes (at 4 months of age) and every month's interval during the first year, thereafter with 3 months' interval. Each value is based on the daily measurements on each rat during 5 days. A, males; B, females.

is evident. Secondly all rats undergo gradual rise of blood pressure except maybe the normotensive females. Thirdly the blood pressure after the start of the experiment does not obviously differ between exposed and non-exposed animals.

Averaged over all measurements, that each group during the first year after the start of the exposure, had pressure for normotensive males was 150, 155 and 145 mmHg for environments A, B, and C respectively. The corresponding values for normotensive females are 130, 125 and 120 mmHg; for the spontaneously hypertensive males, 205, 210, and 215 mmHg; for spontaneously hypertensive females, 200, 205, and 210 mmHg.

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The lack of effect of noise on blood pressure in this experiment may seem surprising in regard to the well-established short-term effects and the earlier presented effects of intermittent noise over longer periods. Three possible explanations can be proposed. First, the

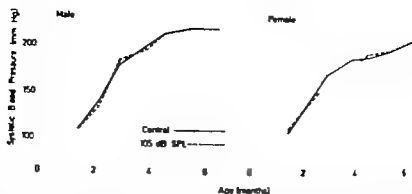


Fig. 1. Average systolic blood pressure for spontaneously hypertensive rats in control environment (—) and 105 dB SPL (---). Arrow indicates start of exposure.

animals have become deaf. Secondly the noise was too weak to give an effect, or outside a hypothetical effective range. Thirdly the rats habituated completely to the noise.

The auditory thresholds for the rats were measured with a behavioral technique (conditioned suppression) after one year in the noise environment. The animals in environment A had a 10–15 dB hearing loss at 6 kHz and those in environment C had a larger loss, 40–60 dB, the value being less for normotensive than for SH rats. In spite of this hearing loss in SH rats, deafness was not the explanation for lack of long-term effects. The second possibility is unlikely since the noise, by giving a hearing loss, evidently is harmful to the rat. The third possibility, habituation, is the most likely explanation for the present lack of effect, and it is supported by a rapid decline of behavioral reaction to the noise.

In conclusion, lifelong exposure, for 10 h daily to the 85 dB SPL or 105 dB SPL noise used in the present study does not alter blood pressure in normotensive or spontaneously hypertensive rats. The present experiments do not exclude the possibility that blood pressure may rise in an environment where noise is not neutral, but a warning signal for noxious events.

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Dopaminergic control of antral gastrin and somatostatin release

By

KRISTIN UYNÄS-WALLÉN, JAN M. LUNDBERG and SUAD ERKÖDÖ

In recent studies in our laboratory concerning the postulated antral inhibitory influence on the acid gastric secretion we perfused antral pouches of conscious dogs with 0.1 M HCl. We then frequently observed an increase of the peripheral gastrin levels. If perfusion had expected a fall of the level of the circulating gastrin on perfusion of the pouch at the low pH. Since we observed that an increase of the gastrin output often preceded with signs of nausea and vomiting we suspected a nervous mechanism behind the increased gastrin release. It was decided to mimic the state of nausea and vomiting by giving apomorphine. I.v. injections of apomorphine (0.01 and 0.1 mg/kg) caused intense vomiting in the dogs and also an increase of the peripheral gastrin levels. Larger amounts of gastrin appeared to be released by the higher dose of apomorphine (Fig. 1 Table I). Apomorphine is supposed to elicit its effects via stimulation of dopamine receptors. The neuroleptic drug haloperidol is considered as a rather specific dopamine receptor blocking drug. When in 4) apomorphine was given 30 min after haloperidol (0.1 mg/kg i.v.) the gastrin release response induced by the lower dose of apomorphine (0.01 mg/kg) was almost abolished while the response to the higher dose (0.1 mg/kg) was reduced to about 50% (Table I). After haloperidol no vomiting or nausea was observed.

In order to characterize further the gastrin release response induced by apomorphine,

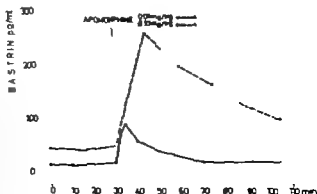


Fig. 1 Release of gastrin following i.v. injection of apomorphine (0.01 and 0.1 mg/kg) in dogs. Note the larger increase of peripheral gastrin levels after injection of the higher dose.

TABLE I Maximal peak increase of peripheral gastrin levels observed in dogs after iv injection of 11 morphine before and after administration of haloperidol 0.1 mg/kg.

Exp. no.	Dose of morphine	Gastrin release response pg/ml		
		Before haloperidol	After haloperidol	Per cent of initial response
1	0.1 mg/kg	70	100	37
2	0.1 mg/kg	180	100	56
3	0.01 mg/kg	105	5	5
4	0.01 mg/kg	85	15	18

this substance was given in acute experiments to cats. On vagal stimulation in cats an gastrin is known to be released not only into the circulation, but also into the antral lumen (Uvnäs-Wallensten and Rehfeld 1976 and Uvnäs Wallensten 1977). Since also somatostatin is released into the antral lumen (Uvnäs-Wallensten *et al.* 1977), we studied the effect of apomorphine on both gastrin and somatostatin release. Acute antral pouches (accomplished by applying ligatures at the antrum-corpus border and the pylorus) were perfused with 0.15 M NaCl pH 6 at a rate of 1 ml/min. After having passed the antrum the perfusates were boiled and analyzed for gastrin and somatostatin like immunoreactivities according to Nilsson (1975) and Elde *et al.* (1978) respectively. Both gastrin and somatostatin were released into the antral lumen by apomorphine 0.01–1 mg/kg. 0.1 mg/kg appeared to release the highest amounts of gastrin and somatostatin (Table II). Atropine 0.1–2 mg/kg failed to inhibit the apomorphine-induced release of these hormones ($n=2$) (Table II).

Thus the present results demonstrate that apomorphine induces an atropine-resistant release of gastrin and somatostatin from the antrum. In the cat electrical vagal stimulation

TABLE II Release of gastrin (G) pg and somatostatin (S) pg ml⁻¹ into the antral lumen of cats following stimulation of apomorphine 0.001–1 mg/kg. The output of hormones was calculated by multiplying the gastrin and somatostatin levels with the volume of the perfusates (10 ml). To obtain the output induced by apomorphine basal output of gastrin and somatostatin was subtracted from the total output observed after administration of apomorphine.

Exp. no.		Apomorphine mg/kg					
		0.001	0.01	0.1	1	0.01	0.1
1	G	0	16 000	60 000	20 000		
	S	0	15 000	60 000	20 000		
2	G	0	1 000	8 000	5 000		
	S	0	2 000	4 000	0		
Atropine 0.2 mg/kg							
3	G		30 000			35 000	
	S		5 000			6 000	
Atropine 2 mg/kg							
4	G		60 000			65 000	
	S		6 000			6 000	

trum releases gastrin via an atropine-resistant mechanism (Uvnäs-Wallensten and Nilsson 1977). Nor can the intraaural vagal release of somatostatin be blocked with atropine (to be published). Apomorphine is known to stimulate central dopaminergic neurons, haloperidol to block them (Andén 1967). To explain these observations it might hence be hypothesized that the increased release of these polypeptides induced by apomorphine is caused by activation of central dopaminergic neurons.

It has been shown that apomorphine also stimulates the release of growth hormone (Larsson *et al.* 1977). Furthermore, Enochs and Johnson (1976) reported that hypophysectomy blocked gastrin release in rats and that the gastrin levels could be restored by injections of growth hormone. At present it cannot be decided whether the release responses observed are secondary to previously released growth hormone, or whether they are mediated via the vagal nerves. As a third possibility apomorphine may activate peripheral dopaminergic receptors. Studies are in progress to investigate the role of the vagus in the apomorphine-induced release of gastrin and somatostatin. Preliminary results indicate that the apomorphine-induced release of gastrin and somatostatin is virtually abolished following vagotomy.

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"Insulinergic" nerves to the skeletal muscles of the cat

By

BÖRJE UVNÄS and KERSTIN UVNÄS-WALLENSTEN

We have recently presented experimental evidence to indicate the distribution of gastrin nerves to the skeletal, smooth and possibly heart musculature of the cat (Uvnäs-Wallensten and Uvnäs 1978). The existence of such extra gastrointestinal sources of gastrin might explain the surprising observation, that in the dog gastrin remains circulating in the blood even after total extirpation of the antrum-duodenum area (Sjödén and Nilsson 1975), which is supposed to contain the major part ($> 90\%$) of the body gastrin. Likewise immunoreactive insulin persists in the blood of eviscerated rats (animals without gastrointestinal tract and pancreas but with intact liver and kidneys) as shown by Smith *et al.* (1978). This similarity in the behaviour of the circulating gastrin and insulin after removal of their main glandular sources, prompted us to look for possible insulinergic nerves as extrapancreatic sources of insulin.

Cats were killed by an i.v. injection of nembutal (0.1 g/kg). Perfusates were collected from eviscerated hind and fore legs from cats perfused with Tyrode's solution via the femoral and brachial artery respectively. For further details of the technique see a concomitant paper in this journal (Uvnäs-Wallensten and Uvnäs 1978). Radioimmunoassay of insulin was performed according to Nilsson (1974).

On electrical stimulation of the sciatic or brachial nerves, insulin-like immunoreactivity appeared in the effluents from the perfused legs, the amounts of immunoreactive material varying with the parameters of the stimuli applied (Table I). Fig. 1 illustrates the characteristic peak-shaped release responses to nerve stimulation. In a concomitant paper (Uvnäs-Wallensten and Uvnäs 1978) we argued for the existence of gastrinergic nerve fibres within the sciatic and brachial nerves. Fig. 2 illustrates the fact that the release of "gastrin" and "insulin" induced by nerve stimulation do not always run in parallel. The perfusates from two hind legs of a cat were concomitantly scanned for gastrin and insulin immunoreactivity. In the one leg (upper curve) stimuli of high voltage (40 V) and long duration (2 ms) caused a marked release of "insulin" with no "gastrin" release response. The lower curve illustrates the opposite result on stimulation of the sciatic nerve to the other hind leg. Stimuli of lower voltage (5 V) and short duration (0.2 ms) induced a marked "gastrin" but no "insulin" outflow. Probably the output of "insulin" and "gastrin" are the results of stimulation of different nerve fibres. As was the case with "gastrin" (Uvnäs-Wallensten and Uvnäs *loc. cit.*) insulin-like material appeared also in perfusates from skinned hind limbs with the circulation of their paws excluded, demonstrating its muscular origin. Prompted by this observa-

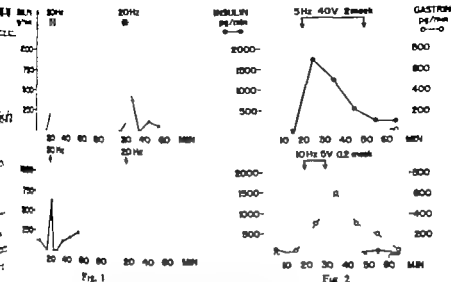


Fig. 1 "Insulin" release following stimulation of the sciatic nerve (upper row) and brachial nerve (lower row) at 5 V 2 ms and 20 Hz. The duration of the stimulations were 5, 3, 1 and 0.5 min. Note the larger release response following stimulations of longer duration.

Fig. 2 "Insulin" and gastrin release following stimulation of the sciatic nerve in two legs. In this experiment stimulation with 5 Hz 40 V and 2 ms induced substantial release of insulin whereas gastrin was released following stimulation with 10 Hz 5 V and 0.2 ms.

we looked for releasable insulin-like material also in the heart muscle. In 2 expts. on the perfused cat heart (Langendorff preparation) insulin-like material appeared on electrical stimulation of the vago-sympathetic trunks.

The chemical identity of the insulin-like material released on nerve stimulation has to await the results of studies in progress. Likewise, the structures to which the immunoreactive material is localized have to be identified. There are receptors in the skeletal muscles capable of binding insulin. Theoretically therefore the "insulin" released on nerve stimulation might emanate from such receptors—or some other so far unknown non-nervous storage structure. In our preparations perfused with Tyrode's solution no visible contractions occurred as the

Table I. Release of insulin from perfused cat legs on stimulation of the sciatic and brachial nerves.

Expt	Hz	V	ms	Duration min	Basal output pg/min	Total release during stimulation (pg)
1	20	15	2	5	0	2300
2	20	15	2	3	0	4300
3	20	15	2	1	50	1400
4	20	15	2	0.5	0	0
5	2	15	2	1	25	6250
6	2	5	2	5	0	8750
7	5	40	2	30	0	35000
8	20	20	2	5	0	15900

result of nerve stimulation. The release of the insulin-like material therefore does not seem to be secondary to the contraction process. To us one possible source of the released material might be "insulinergic" nerves. Even if the acceptance of the existence of such nerves requires biochemical and immunochemical identification of insulin in the nerves supplying muscles, some speculations on the functional significance of possible "insulinergic" nerves might be justified. We have so far only experimental evidence suggesting such fibres to skeletal muscles and possibly the heart. The purpose of an "insulinergic" innervation might be to adapt, via locally released insulin, the energy supply of the muscles to their degree of contractile activity. Such fibres might supply also smooth muscles, glands etc. A more precise definition of the functional role of the "nervous insulin" as well as the mechanism of activation of the "insulinergic" nerves have to await further detailed studies.

The possible clinical significance of "insulinergic" nerves for our understanding of the nature of diabetes and other metabolic diseases remains to be clarified.

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Release of gastrin on stimulation of the sciatic and brachial nerves of the cat

By

KERSTIN UVNÄS-WALLENSTEN and BÖRJE UVNÄS

The stomach and the upper part of the duodenum harbour the overwhelming part of the gastrin in the gastrointestinal tract of cat, dog, hog and human (Emlis and Fyrb 1968, Uvnäs *et al.* 1973). Electrical stimulation of the cat's vagi causes release of gastrin (identified as heptadecapeptide) concomitantly into the portal blood and the antral lumen (Uvnäs-Wallensten 1977). The vagal fibres responsible for the gastrin release seem to be non-cholinergic since the release response was uninfluenced by atropine (Uvnäs-Wallensten and Andersson 1977). Our assumption that the vagi might contain gastrinergic fibres was supported by the finding of gastrin heptadecapeptide in extracts from the vagi of cats, dogs and humans (Uvnäs-Wallensten *et al.* 1977). Attempts to visualize vagal gastrinergic fibres by immunofluorescence techniques revealed such fibres in the muscular layers of the stomach (Lundberg and Hokfelt personal communication). Gastrinergic nerves have also been demonstrated in the colon (Uvnäs-Wallensten *et al.* 1977). Apparently gastrointestinal smooth muscles have a gastrinergic nerve supply. With the idea that a gastrinergic innervation of smooth muscles might reflect a more general biological phenomenon we decided to look for gastrinergic nerves to other types of muscles.

The present experiments were performed *in vivo* on hind and fore limbs of cats. The animals were killed with sodium pentobarbital, the cutaneous nerves exposed above the knee and the elbow respectively and perfused with Tyrode solution at room temperature at a rate of 1 ml/min. The perfusate was collected in tubes surrounded by ice. Samples, taken at 2 to 10 min intervals were immediately boiled for 1 min to 100°C before being frozen and stored. The sciatic or brachial nerves were stimulated by Grass stimulator; the duration, frequency and intensity of the impulses being varied as desired (Figure and table). The perfusate samples were assayed for immunoreactive gastrin with radioimmunoassay described by Nilsson (1975).

On nerve stimulation immunoreactive material appeared in the perfusates from both hind and fore limbs (Table 1), the amounts released being dependent on the parameters of the stimuli applied (Fig. 1). The release occurred as a single shortlasting peak (around 5-10 min), in spite of continuous stimulation. To exclude perfusion of the skin, in two cats the hind legs were skinned above the ankle and the circulation in the paws excluded by tight ligatures. Immunoreactive material still appeared in the perfusates (Fig. 2), indicating its vascular origin. In three experiments immunoreactive material appeared in blood collected from the peripheral vein, or in blood from the external facial vein on stimulation of the sciatic and

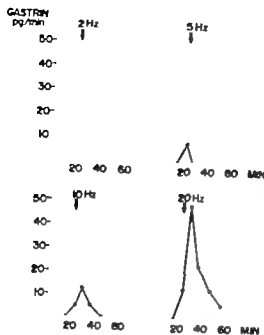


Fig. 1

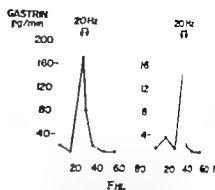


Fig. 2

Fig. 1 Occurrence of gastrin-like material in furates from hind (lower row) and fore (upper row) of cat on stimulation of the sciatic and brachial nerves respectively. Stimulation parameters: 15 V, ms, 5, 10 and 20 Hz. Note increasing release response with increasing frequency.

Fig. 2 Occurrence of gastrin-like material in furates from the skinned hind limb of a cat the paw excluded by metal wire ligatures. Stimulation parameters: 20 V, ms, 20 Hz.

the facial nerve respectively (Table I). In the 2 experiments performed, gastrin-like material appeared in the popliteal venous blood on stimulation of the ventral roots L₅ and S₁.

Since no gastrin cells are known to occur in skeletal muscles we assume the gastrin-like immunoreactive material released on nerve stimulation to originate from gastrinergic nerves although a localization to other gastrin-containing structures cannot be ruled out. Attempts are presently made to visualize the muscle gastrin stores with immunofluorescence technique as also to identify the gastrin variant responsible for the immunoreactivity.

The possible occurrence of gastrinergic nerves to both smooth and striated muscle prompted us to look for such nerves also in heart muscle. In two preliminary experiments on the isolated perfused cat heart (a Langendorff preparation) stimulation of the vagosympathetic trunks resulted in the appearance of immunoreactive material (to be published).

The physiological role of a possible gastrinergic innervation of the muscles can at present only be speculated on. It is reported that long term administration of pentagastrin to pregnant dogs and newborn puppies leads to hypertrophic pyloric stenosis in the pups (Dodge, 1970). The gastrinergic nerves might therefore influence the contractile machinery or have some "trophic" influence on muscles. Since gastrin administration also has been reported to result in hypertrophy of the parietal cell mucosa of the rat (Johnson, 1974) one might imagine gastrin as a hormonal and nervous "trophic" or "stimulating" factor of general biological significance, influencing growth (and) or activity in muscles, glands and other metabolically active tissues and cells.

The antrum-duodenum area is assumed to be the main source of circulating gastrin. This gastrin has a rapid turn-over with half lives of the various gastrin components of <30 min. It has therefore been surprising to find gastrin remaining in the blood after extirpation of the antrum-duodenum in dogs (Sjödén and Nilsson, 1975). One reason for the persistence

ml) Occurrence of gastrin-like material in perfusates and blood on electrical stimulation of the sciatic nerve, the brachial nerve, the facial nerve, and ventral roots L₁, S₁ and S₂.

No	Stimulation parameters				Basal release pg/min	Total release during stimulation (pg)
	Rz	V	am	Duration min		
Values from corpora cat bodies						
1	20	20	2	5	10	1 300
2	20	20	2	5	2	50
3	20	20	2	5	2	30
4	2	15	2	1	0	0
5	5	15	2	1	0	40
6	10	15	2	1	0	120
7	20	15	2	1	0	700
8	20	15	2	5	0	1 000
9	20	20	2	5	0	1 800
10	2	5	2	5	0	75
11	10	5	2	5	0	200
12	10	15	0.2	5	0	4 000
Values from lead iv in vivo (stimulation of sciatic nerve)						
13	20	0.1	5		5 (pg/ml)	1 000
Values from central facial root (stimulation of facial nerve)						
14	20	2	5		7 (pg/ml)	150
Values from lead iv in vivo (stimulation of L ₁ , S ₁ , S ₂)						
15	20	10	0.5	5	20 (pg/ml)	400
16	20	10	2	5	30	300

of gastrin in the blood after removal of these main gastrin stores might be the release of gastrin from peripheral gastrinergic nerves.

1411 Prof Gastrin in both perfusates has been identified as gastrin 17 by Gregory and Dockray (personal communication) and visualized in sciatic nerve fibres by Lundberg and Holte (personal communication).

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ADDENDUM to Ca^{++} sensitive microelectrode: intracellular steady state measurement in nerve cell

BY

G. R. J. CHRISTOFFERSEN and LEIF SIMONSEN

Following the publication in *Acta physiol. scand.* 101 491-494 of the communication entitled Ca^{++} sensitive microelectrode intracellular steady state measurement in nerve cell we have received a comment from Dr J. D. Owen, Dept. of Physiol., Univ. of U. Medical Center and Prof. H. Mack Brown, same address, regarding our reference to article A Calcium-sensitive microelectrode suitable for intracellular measurement Calcium (II) activity H. M. Brown, J. P. Pemberton and J. D. Owen, (1976) *Anal. Chim. Acta* 85 261-276. The authors find that—to pay proper tribute to their contributions—the reference to their article should have been placed in relation to the following paragraph in our communication.

"The electrode was prepared as described by Christoffersen and Johansen (1976) except for two changes. The PVC content was 10% instead of 40% and the external tip diameter was 1 μm instead of 20 μm .

It was found in our laboratory before the publication of the last mentioned article that reduction of tip size of the previously described electrode containing 40% PVC (Christoffersen and Johansen 1976) towards 1 μm decreased the slope of calibration plots and increased electrode resistance and response time beyond practical use, and further that a reduction of PVC content to 10% counteracted these effects. Before submission to publication of our manuscript concerned with intracellular use in neurons of *Helix pomatia* the effects of PVC content and tip size on slopes of calibration plots were published (Brown, Pemberton and Owen 1976). We therefore confirm what may be read from the reference cited in our communication that the first published description of these effects is found in the article (Brown, Pemberton and Owen 1976).

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CHRISTOFFERSEN, G. R. J. and L. S. JOHANSEN. Microdesign for a calcium-sensitive electrode *Anal. Chim. Acta* 1976, 81 191-193.

Effect of intratubular substitution of Na⁺ and Cl⁻ ions on the operation of the tubuloglomerular feedback

By

R. MÖLLER-SAUR and H. U. GUTSCHE

Received 10 February 1976

Abstract

MÖLLER-SAUR, R. and H. U. GUTSCHE. *Effect of intratubular substitution of Na⁺ and Cl⁻ ions on the operation of the tubuloglomerular feedback* Acta physiol. scand. 1978. 103. 353-362.

Renal loops of single nephrons in rat kidneys were perfused orthogradely with various solutions of different compositions, while the stop-flow pressure (SFP) was monitored as the early proximal tubular measure of the tubuloglomerular feedback response. Modified Ringer solution or iso-osmotic solutions of NaCl, KCl, LiCl, RbCl, choline-Cl, and Na-acetate used to perfuse Henle loop led to significant SFP decrease indicating an intact operation of tubuloglomerular feedback mechanism. Increased rates of iso-osmotic solutions of sodium sulphate, potassium sulphate and acetazolol did not yield SFP alterations. In order to estimate intratubular sodium and chloride concentration at the macula densa during Ringer, LiCl, choline-Cl, and Na-acetate perfusion, early distal tubular fluids were collected at low and high perfusion rates, corresponding to an absent or maximal feedback response. Analysis was performed by microfluorimetry and microchloride titration. The results showed that there does not exist a response threshold for the early distal sodium or chloride concentration at which the feedback starts to operate. We conclude that the intraluminal signal at the macula densa, mediating a tubuloglomerular feedback response, was secretarily coupled to single ion species. Either an unspecific ion signal or some picture events of ionic interaction with the macula densa cells might operate as the initiating step in the feedback loop.

Key word: Rat kidney single nephron GFR, stop-flow pressure, micropertfusion, loop of Henle, macula densa, tubuloglomerular feedback.

The existence of a tubuloglomerular feedback mechanism, first demonstrated by Thirau and Schniermann (1965) has been confirmed by several laboratories (Hjerholzer et al. 1972, Isack, Rector and Seidlin 1973, Schniermann et al. 1970, Schniermann, Persson and Ågerup 1973, Wright and Schniermann 1974). However single steps of this feedback mechanism are still under investigation. Much controversy exists on the specificity of the triggering factor(s). Originally Thirau and Schniermann (1965) postulated the intratubular sodium concentration at the macula densa as the initiating signal. In consecutive publications rather the sodium chloride transfer through the macula densa cells than the intraluminal concentra-

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Effect of intratubular substitution of Na⁺ and Cl⁻ ions on the operation of the tubuloglomerular feedback

By

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Received 10 February 1976

Abstract

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Paired loops of surface nephrons in rat kidneys were perfused orthogradely with various solutions of different compositions, while the stop-flow pressure (SFP) was monitored in the early proximal tubulus as an index of the tubuloglomerular feedback response. Modified Ringer solution or iso-osmotic solutions of NaCl, KCl, LiCl, RbCl, choline-Cl, and Na-acetate used to perfuse Henle loop led to significant SFP decrease indicating an intact operation of tubuloglomerular feedback mechanism. Increased rates of iso-osmotic solutions of sodium sulphate, potassium sulphate and mannitol did not yield SFP alterations. In order to examine intratubular sodium and chloride concentration at the macula densa during Ringer, LiCl-choline-Cl- and Na-acetate perfusions, early distal tubular fluids were collected at low and high perfusion rates, corresponding to an absent or maximal feedback response. Analysis was performed by ion-selective electrodes and microchloride titration. The results showed that there does not exist a narrow threshold for the early distal sodium or chloride concentration at which the feedback starts to operate. We conclude that the intratubular signal at the macula densa, initiating tubuloglomerular feedback response is not necessarily coupled to single ion species. Either an unspecific ion transfer or some passive events of some interaction with the macula densa cells might operate as the initiating step in the feedback loop.

Key words: Rat kidney single nephron GFR, stopflow pressure, micropuncture, loop of Henle, macula densa, tubulo-glomerular feedback.

The existence of a tubuloglomerular feedback mechanism, first demonstrated by Thoreau and Schnermann (1965) has been confirmed by several laboratories (Hjerholzer *et al.* 1972, Iwata, Rector and Selkio 1973, Schnermann *et al.* 1970, Schnermann, Persson and Ågerup 1973, Wright and Schnermann 1974). However single steps of this feedback mechanism are still under investigation. Much controversy exists on the specificity of the triggering factor(s). Originally Thoreau and Schnermann (1965) postulated the intratubular sodium concentration at the macula densa as the initiating signal. In consecutive publications rather the sodium chloride transfer through the macula densa cells than the intraluminal concentra-

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Table 1 Compiled data (mean \pm S.E.) of stop flow pressure changes during perfusion of Henle loop with different solutions at rates between 0-30 ml/min. Significance of differences are calculated with SFP during zero perfusion, 0.05 to 0.001 means $P < 0.05$ to < 0.001 and errors Ringer perfusion (vertical columns), +0.02 to 0.001 means $P < 0.02$ to < 0.001 - numbers of nephrons which the corresponding perfusion rate was tested.

Nephron	Number of nephrons	SFP at zero loop perfusion [cmH ₂ O]	Δ SFP \pm				
			10 ml/min [cmH ₂ O]	20 ml/min [cmH ₂ O]	30 ml/min [cmH ₂ O]	40 ml/min [cmH ₂ O]	50 ml/min [cmH ₂ O]
1 Ringer	18	11.5 ± 2.2	-2.0 ± 1.3 -10	-12.3 ± 1.5 13 +0.001	13.7 ± 1.7 -11 +0.001	-10.8 ± 1.4 -6 +0.01	-11.2 ± 0.9 12 +0.001
2 NaCl	3	40.0 ± 0.9	± 0 -2	-6.0 -2	-10.3 ± 2.3 -3 +0.05	—	-9.5 -1
3 LiCl	8	30.3 ± 3.0	0.5 ± 0.7 5	-9.2 ± 1.2 -3 +0.001	-15.2 ± 3.1 6 +0.01	-16.5 ± 3.0 4 +0.02	-15.8 ± 3.7 6 +0.02
4 KCl	6	11.9 ± 1.1	± 0 -1	-2.0 -2	7.4 -2	-10.0 2	-8.8 ± 0.9 -5 +0.001
5 K ₂ Cl	2	43.7	0.5 -1	-3.5 2	8.5 2	13 1	-10.5 2
6 Calcium Cl	6	48.5 ± 1.9	-2.8 ± 1.4 3	-6.0 -2	6.7 ± 1.8 4 +0.05 +0.02	-6.6 ± 2.1 4 +0.05	6.4 ± 1.5 -6 +0.02 +0.02
7 K ₂ -ascorbate	6	42.4 ± 2.7	-0.8 ± 0.6 -5	-7.6 ± 2.8 4	-10.3 ± 0.3 -3 +0.001	-9.5 -1	-9.7 ± 0.5 -6 +0.001
8 Na ₂ SO ₄	4	64.9 ± 11.2	$+0.4 \pm 0.9$ 3	$+1.5$ 8-2	0.4 ± 0.5 3 0.001	-2.5 ± 1.2 -4 +0.01	-0.7 ± 0.9 -3 +0.001
9 K ₂ SO ₄	3	54.0 ± 2.4	—	—	+1 -1	-1.2 -1	$+0.7$ 2
10 Mannitol	11	48.5 ± 2.9	-1 2	-1.5 ± 0.7 5 +0.001	-1.4 ± 1.0 5 0.001	$+4.5 \pm 1.8$ -3 +0.001	-0.3 ± 0.9 -10 0.001

maintained at 0.2% leucostatin stained solutions. No apparent influence on the feedback response was observed.

For distal collection expts. rats were prepared as for the SFP expts. The loop of Henle was perfused from an end-proximal site downstream to a paraffin block to mid-proximal tubule to prevent ultrafiltrate admixture. Collections of tubular fluid from early distal segments were performed using pipettes with 6-10 μ m tips filled with stained castor oil. The collection site could be identified by the coloured perfusion fluid. Timed samples were taken proximal to a mobile castor oil block from early distal segments. The suction of the collecting pipette was adjusted so that a constant perfusion pressure was measured at the late proximal site. If possible two collections from one nephron were performed at low

tion was defined as the feedback signal (Schnermann *et al.* 1970 Wright and Schnermann 1974). In experiments of Israelit Rector and Seldin (1973) and Burke *et al.* (1974) the intraluminal Ca^{2+} activity seemed to play a crucial role, which, however, could not be confirmed by others (Schnermann and Ploth 1975). The most recent hypothesis by Wright and Person (1974) and Schnermann Ploth and Hermle (1976) is that feedback responses depend critically upon the rate of chloride transport across or into the macula densa cells. In our first experiments (Müller-Suur, Gutsche and Hegel 1973) on this problem we could not detect a distinct ionic specificity of the feedback mechanism. The present experiments further analyse the ionic requirements for the sensor of this feedback mechanism and try to examine if there is a distinct luminal concentration of sodium or chloride at which the mechanism starts to operate. Our results show that the feedback regulation of stop-flow pressure is not specifically dependent on changes in sodium or chloride concentrations at the macula densa and that substitution of sodium and chloride by some unphysiological ions left the feedback loop intact.

Methods

Microperfusion experiments were performed on male Wistar rats (Fa. Winkelmann, Hannover, W. Germany) weighing 150–250 g, kept on Altromin R (Altromin GmbH, Lage-Lippe, W. Germany) diet as described earlier (Müller-Suur *et al.* 1975). Anaesthesia by i.p. Inactin® (Promonta, Hamburg), 100 mg/kg B.W., i.v. infusion of 0.9% NaCl, starting dose 1 ml immediately after the preparation followed by a sustaining rate of 17 ml/h. Under these conditions all animals were antidiuretic. Arterial blood pressure was monitored via the left carotid artery catheter using a pressure transducer (Bell and Howell, type 4-327-L233, Barinstoke, U.S.A.). The urethra was cannulated (polyethylene PE 50 catheter) and timed urine samples were collected under mineral oil. The left kidney was immobilized in a thermoregulated metal chamber (37°C) and covered with prewarmed mineral oil. The experimental protocol for the tubuloglomerular feedback response consisted of measuring the stop-flow pressure of the early proximal tubules related to the pressure controlled microperfusion of the loop of Henle at rates between 0 and 50 nl/min, as described in detail earlier (Müller-Suur *et al.* 1975).

The equipment for microperfusion, i.e. pressure transducer (Kulite, CPL series) and microperfusion system and the set up for tubular blockade with solid paraffin is described elsewhere (Gutsche *et al.* 1975 and Hierholtzer *et al.* 1974). The following solutions were used for microperfusion of Henle's loop: 1. modified Ringer's solution (130 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 10 mM NaHCO_3 , 7 mM urea, 0.3 g/100 ml indigo-carmin blue, buffered with TRIS to pH 7.4); 2. NaCl (150 mM NaCl, 0.3% indigo-carmin blue); 3. KCl (150 mM KCl, 0.3% indigo-carmin blue); 4. LiCl (169 mM LiCl, 0.3% indigo-carmin blue); 5. RbCl (165 mM RbCl, 0.3% indigo-carmin blue); 6. Choline-Cl (150 mM choline-Cl, 0.3% indigo-carmin blue); 7. Na-acetate (150 mM Na-acetate, 0.3% indigo-carmin blue); 8. Na_2SO_4 (91 mM Na_2SO_4 , 75 mM mannitol, 0.3% indigo-carmin blue); 9. Mannitol (300 mM mannitol, 0.3% indigo-carmin blue). Mean osmolality of all solutions was 310 mosm/kg. In some pilot expts. the influence of staining of the perfusate was tested by using

TABLE 1 Compiled data (mean \pm S.E.) of stop flow pressure changes during perfusion of Henle loop with different substances at rates between 0-30 ml/min. Significance of differences were calculated versus SFP during zero perfusion, 0.05 to 0.001 means $P < 0.05$ to < 0.001 and versus Ringer perfusion (vertical column), +0.02 to 0.001 means $P < 0.02$ to < 0.001 - numbers of nephrons at which the corresponding perfusion rate was tested.

Substance	Number of nephrons	SFP at zero loop perfusion [cmH ₂ O]	Δ SFP at 10 ml/min [cmH ₂ O]	20 ml/min [cmH ₂ O]	30 ml/min [cmH ₂ O]	40 ml/min [cmH ₂ O]	50 ml/min [cmH ₂ O]
1 Ringer	10	31.5 \pm 2.2	-2.0 \pm 1.3 -10	-12.3 \pm 1.3 11 *0.001	-13.7 \pm 1.7 -11 *0.001	-10.8 \pm 1.6 6 *0.01	-11.2 \pm 0.9 12 *0.001
2 NaCl	3	60.0 \pm 0.9	\pm 0 2	6.0 2	-10.3 \pm 2.3 -3 *0.05	—	9.5 -1
3 LiCl	6	50.3 \pm 3.0	0.5 \pm 0.7 5	-9.2 \pm 1.2 -3 *0.001	15.2 \pm 3.1 8 *0.01	-16.5 \pm 3.0 4 *0.02	15.0 \pm 3.7 -6 *0.02
4 KCl	6	57.9 \pm 1.1	\pm 0 -1	-2.0 -2	7.4 2	-10.0 -2	-8.8 \pm 0.9 5 *0.001
5 NaCl	2	43.7	0.5 1	-3.5 2	8.5 2	-13 -1	-10.5 2
6 Choline Cl	6	48.5 \pm 1.9	2.8 \pm 1.4 -3	-6.0 -2	6.7 \pm 1.8 -4 *0.05 0.02	-6.6 \pm 2.1 -4 *0.05	6.4 \pm 1.5 -6 *0.02 +0.02
7 Na-acetate	6	42.4 \pm 2.7	0.8 \pm 0.6 -5	-7.4 \pm 2.8 4	-10.3 \pm 0.3 -3 *0.001	9.5 1	-9.7 \pm 0.5 -6 *0.001
8 Na ₂ SO ₄	4	64.9 \pm 11.2	+0.4 \pm 0.9 -3	+1.5 -2	-0.4 \pm 0.5 3 +0.001	-2.5 \pm 1.2 4	-0.7 \pm 0.9 -3 0.001
9 K ₂ SO ₄	3	54.0 \pm 2.4	—	—	+1 1	-1.2 1	+0.7 -2
10 Mannitol	11	48.5 \pm 2.9	-1 -2	-1.5 \pm 0.7 -5 +0.001	-1.6 \pm 1.0 5 0.001	+4.5 \pm 1.5 3 +0.001	-0.3 \pm 0.9 -10 0.001

was used or 0.2% Massarin stained solutions. No apparent influence on the feedback response was observed.

For data collection expts. rats were prepared as for the SFP expts. The loop of Henle was perfused from an end-proximal site downstream to a paraffin block in a mid-proximal tubule to prevent ultrafiltrate admixture. Collections of tubular fluid from early distal segments were performed using pipettes with 6-10 μ m tips filled with stained castor oil. The collection site could be identified by the coloured perfusion fluid. Timed samples were taken proximal to a mobile castor oil block from early distal segments. The suction of the collecting pipette was adjusted so that a constant perfusion pressure was measured at the late proximal site. If possible two collections from one nephron were performed at low

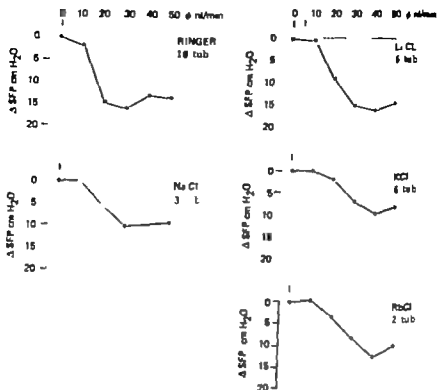


Fig. 1 SFP changes during perfusion of Henle's loop with isotonic Ringers, NaCl, LiCl, KCl, and RbCl. For means \pm S.E. see Table I.

(10–15 nl/min) and high (50–75 nl/min) perfusion rate. The first collection was randomly done at high or low perfusion rate. Each sample was transferred under water saturated mineral oil and analysed for Na⁺ using a microflame photometer (Malnic, Klose and Gebisch 1964) and for chloride by galvanometric titration (Ramsay Brown and Croghan 1955).

In expts. with sodium acetate another approach of the feedback analysis was made. Single nephron GFR (SNGFR) and early proximal flow rate (EPFR) was measured proximal to a solid paraffin block during perfusion of Henle's loop with Na-acetate (at rates of 0 and 60 nl/min). SNGFR and EPFR were calculated from the collected volumes measured in constant bore pipettes and tubular fluid to plasma inulin ratio counting the radioactivity of ³H-inulin in the tubular fluid sample and artery plasma samples. In these expts. ³H-inulin was infused at a rate of 75 μ Ci/h (50 μ Ci/ml).

The tables compile mean values and \pm S.E. significance of differences were calculated with the student's test. The SFP at zero perfusion and the Δ SFP at different perfusion rates through Henle's loop were averaged for each nephron from repetitive measurements. Mean observation time in each nephron was 42 min and during this interval the feedback response was elicited 5 to 10 times.

Results

Stop-flow pressure feedback studies

Feedback response by a decreased proximal SFP was obtained at increased flow rate through the loop of Henle with solutions 1–7 whereas perfusion with Na₂SO₄, K₂SO₄ and mannitol

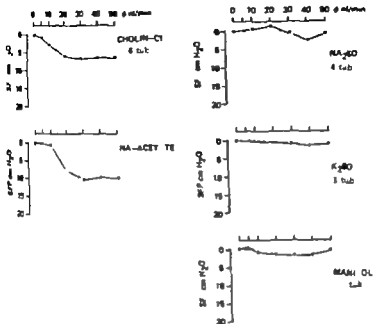


Fig. 2 EPFR changes during perfusion of Henle loop with osmotic diuretic: CHOLINE-Cl, Na-acetate, Na₂SO₄, K₂SO₄, and mannitol. For means \pm S.E. see Table I.

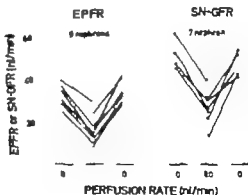
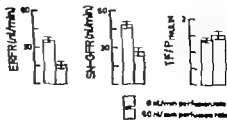


Fig. 3 Early proximal flow rate (EPFR) and single nephron filtration rate (SN-GFR) and tubular fluid to plasma ratio of sodium (TF/P_{Na}) during perfusion of Henle loop with osmotic diuretic solution at rates of 0 and 60 ml/min. Above: single experiments' data, below: mean values \pm S.E. significantly different ($P < 0.05$).



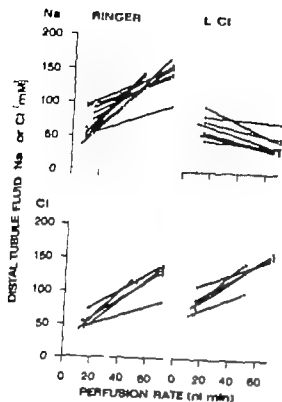


Fig. 4

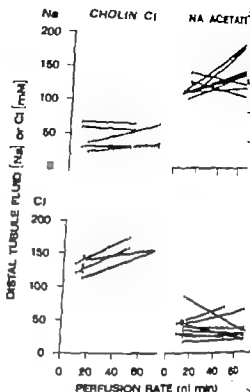


Fig. 5

Fig. 4. Na^+ and Cl^- concentration in early distal tubular fluid collected at different rates of perfusion through Henle's loop with isosmotic Ringer's and LiCl solution. Lines indicate measurements in the same nephron.

Fig. 5. Na^+ and Cl^- concentration in early distal tubular fluid collected at different rates of perfusion through Henle's loop with isosmotic choline- Cl and Na -acetate solution. Lines indicate measurements in the same nephron.

had no influence on SFP. The data are compiled in Table I. The results are depicted in Fig. 1 and 2. Compared with Ringer's the LiCl perfusion (see Fig. 1), yielded slightly but not significantly greater ΔSFP at 50 nl/min loop perfusion; whereas choline- Cl at this rate showed a significantly lower ΔSFP compared to Ringer's. From these figures it becomes further evident that the onset of the feedback response starts above perfusion rates of 10 nl/min, maximal response was attained between 20 nl/min to 50 nl/min. This behavior typical for Ringer's was also apparent in the other perfusion experiments (Fig. 1 and 2).

EPFR and SNGFR measurement during Na -acetate feedback

In 9 nephrons EPFR and SNGFR was measured during Na -acetate induced feedback response or at the state of no feedback stimulus, i.e. zero perfusion of the loop. Fig. 3 shows the single measurements and the mean \pm S.E. Under orthograde loop perfusion with Na -acetate EPFR and SNGFR decreased to about 50% of the value under zero perfusion. TF/P inulin ratio slightly increased from a mean of 1.3 ± 0.05 to 1.5 ± 0.1 . Thus, the observed SFP decreases (Fig. 2) were associated with decreases also of EPFR and SNGFR (Fig. 3).

TABLE II. Na^+ and Cl^- concentrations in early distal tubular fluid collected during perfusion of Henle loop from subpyramidal site downstream solid paraffin block at different perfusion rates (nl/min). 0.02-0.001 gives significance of difference from values at 10-15 nl/min perfusion ($P < 0.001$) and + gives significance of difference from corresponding values with Ringers perfusion (vertical column) at $P < 0.001$. — number of nephrons.

Perfusion at	mM Na^+		mM Cl^-	
	10-15 nl/min	50-75 nl/min	10-15 nl/min	50-75 nl/min
Ringer	73.9 \pm 4.9 14	139.0 \pm 6.0 14 *0.001	49.3 \pm 4.7 8	122.5 \pm 5.6 9 *0.001
LiCl	71.3 \pm 3.6 11	43.6 \pm 3.1 9 *0.01	82.8 \pm 6.2 6	144.5 \pm 10.1 5 *0.001
Choline-Cl	32.1 \pm 5.2 10	40.1 \pm 5.0 11	126.2 \pm 5.1 9	160.0 \pm 3.8 6 *0.001
Na-acetate	+	+	+	+
	114.5 \pm 5.5 10	144.8 \pm 10.0 10 *0.02	41.8 \pm 5.9 10	30.6 \pm 3.7 12
	+			+

Fluid collection experiments

In a second set of expts. we studied the change of ionic composition of early distal tubular fluid during perfusion of Henle's loop with Ringer, LiCl, choline-Cl and Na-acetate. Increased flow rate of these solutions induced a feedback response (see Fig. 1, 2 and 3). Elevated perfusion rates increased early distal sodium concentration in the case of Ringer and Na-acetate perfusion. The early distal chloride concentration increased in the case of high perfusion rate with Ringer, LiCl and choline-Cl. Single data are given in Fig. 4 and 5 and mean \pm S.E. are compiled in Table II. It is also apparent from these expts. that an increase of LiCl-perfusion led to a decrease of the distal sodium concentration. During different Na-acetate perfusion rates the distal chloride concentration did not change significantly.

Discussion

The present expts. were designed to define further the chemical composition of the tubular fluid necessary to obtain a feedback response. For this reason loops of Henle were perfused with solutions of different ionic compositions either Na^+ or Cl^- free under continuous recording of early proximal stop flow pressure. It could be shown that during perfusion with iso-osmotic NaCl, LiCl, KCl, RbCl, choline-Cl and Na-acetate an intact tubuloglomerular feedback response was observed as found earlier in expts. with Ringer's perfusion. Secondly this feedback response could be elicited in the same tubulus during more than 1 hour of repeated stimulation and started only when the perfusion was above 10 nl/min being maximal between 20 and 50 nl/min. Thirdly perfusion with 50 containing

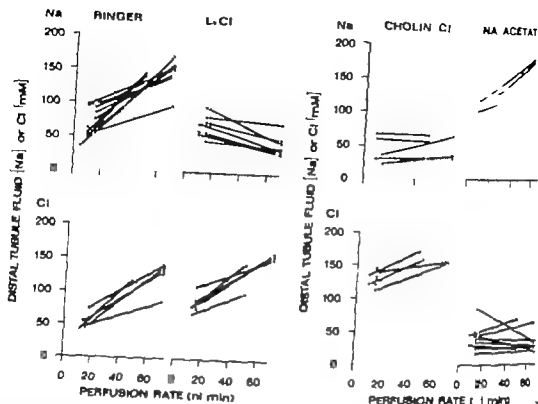


Fig. 4

Fig. 4 Na^+ and Cl^- concentration in early distal tubular fluid collected at different rates of perfusion through Henle's loop with isotonic Ringer's and LiCl solution. Lines indicate measurements in the same nephron.

Fig. 5 Na^+ and Cl^- concentration in early distal tubular fluid collected at different rates of perfusion through Henle's loop with isotonic choline- Cl and Na -acetate solution. Lines indicate measurements in the same nephron.

Fig. 5

had no influence on SFP. The data are compiled in Table I. The results are depicted in Fig. 1 and 2. Compared with Ringer's the LiCl -perfusion (see Fig. 1) yielded slightly but not significantly greater ΔSFP at 50 nl/min loop perfusion, whereas choline- Cl at this rate showed a significantly lower ΔSFP compared to Ringer's. From these figures it becomes further evident that the onset of the feedback response starts above perfusion rates of 10 nl/min, maximal response was attained between 20 nl/min to 50 nl/min. This behavior typical for Ringer's was also apparent in the other perfusion experiments (Fig. 1 and 2).

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In 9 nephrons EPFR and SNGFR was measured during Na -acetate induced feedback response or at the state of no feedback stimulus, i.e. zero perfusion of the loop. Fig. 3 shows the single measurements and the mean \pm S.E. Under orthograde loop perfusion with Na -acetate EPFR and SNGFR decreased to about 50% of the value under zero perfusion. The TF/P inulin ratio slightly increased from a mean of 1.3 ± 0.05 to 1.5 ± 0.1 . Thus, the observed SFP decreases (Fig. 2) were associated with decreases also of EPFR and SNGFR (Fig. 3).

Thus, the feedback response is more likely to be initiated by a passive mechanism in the ascending limb. The results of Wright and Persson (1974) that transepithelial hyperpolarization produced a feedback response (Wright and Persson, 1974) can also be in agreement with the latter explanation. Morphological support of the hypothesis of passive processes associated into the feedback stimulation can be derived from the studies of Latta, who demonstrated that the morphology of the macula densa cells is different compared to the epithelial cells of the ascending limb and the early distal tubulus epithelium (Latta 1974). Furthermore, Betts et al., Shahood and Rosen (1975) demonstrated the absence of transport enzymes such as Na-K ATPase in macula densa cells. They concluded that the osmotic receptor function is not compatible with an active NaCl transport process. Recently in micropuncture studies by Navar and Bell (1977) these authors suggested that the passive osmolarity may induce the feedback response.

Transmembrane or transepithelial potential difference changes in dependence of the tubular ionic strength could be one of possible passive steps initiating the tubuloglomerular feedback response. However the present studies cannot discriminate between the two mentioned concepts. Direct experiments on surface macula densa cells and further histological analyses of single macula densa cells are needed to further clarify the mechanism.

Technical assistance is given by L. Lichtenstein, S. Lidenitz and H. Seibe

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solutions (sodium and potassium sulphate) and mannitol did not lead to a feedback reaction. These findings are in agreement with reports in the literature (Schnermann, Persson and Ågerup 1973, Schnermann and Hermle 1975, Wright and Schnermann 1974) with the exception of studies with Na-acetate perfusion. Schnermann, Plöth and Hermle (1976) did not observe a feedback response under orthograde Na-acetate perfusion concluded from EPFR recollection expts. Comparing their results with ours the authors argued that parallel changes of stop flow pressure and EPFR need not occur under all experimental conditions.

However in earlier studies no such discrepancies between changes of SNGFR, EPFR and SFP was found (Hierholzer *et al.* 1974, Müller Suur *et al.* 1975, Schnermann *et al.* 1970, Wright and Schnermann 1974). Also in the present study of Na-acetate perfusion a high flow rate induced parallel changes of SNGFR, EPFR and SFP. From our results it can be shown that Na-acetate releases a tubuloglomerular feedback response. As Schnermann, Plöth and Hermle (1976) used EPFR measurements only it seems possible that by changes of fractional reabsorption *Le.* T/FP inulin, actual changes in SNGFR remained undetected.

Distal collection expts. at early distal puncture sites in some of the present perfusion studies were performed to analyse changes of the ionic composition under perfusion through Henle's loop. Although an increase of perfusion with Ringer's LiCl, choline-Cl and Na-acetate led in all cases to a feedback stimulated SFP-decrease, an elevated distal Na⁺-concentration was found only with Ringer's and Na-acetate, whereas no change of distal Na⁺-concentration was seen with choline-Cl perfusion and a rather decreased Na⁺-concentration with LiCl perfusion (see Fig. 4 and 5 and Table II). Together with the fact that under Na₂SO₄ perfusion no feedback response was observed despite increasing distal Na⁺-concentration (Schnermann *et al.* 1970) this ion seems not necessarily be required for the feedback activation.

The role of the early distal Cl⁻ concentration changes within the feedback loop is not similarly conclusive from the present expts. However likewise no distinct Cl⁻ concentration can be postulated from our expts. at which the mechanism starts to operate. Absent feedback response at low perfusion rates can be associated with high distal Cl⁻ (choline-Cl and LiCl) or low distal Cl⁻ (Ringer's, Na-acetate). Likewise states with maximal feedback response can be accompanied by low (Na-acetate) and by high distal Cl⁻ concentrations (Ringer's, choline-Cl, LiCl). Thus, the chloride concentration per se cannot be the triggering factor of the feedback mechanism.

Thus, the nature of the flow dependent signal initiating the tubuloglomerular feedback response is yet not clarified. However there exist at least two distinct possibilities. Wright and Persson (1974) and Schnermann, Plöth and Hermle (1976) suggested that a supposed transport of Cl⁻ (Burg *et al.* 1973) or at least of a halide by macula densa cells is mediating the feedback mechanism. The present expts. in addition are in agreement with the assumption that even acetate anions are included into this combined anion transport-receptor mechanism. Recent studies of Gutsche *et al.* on the transport processes of the ascending limbs of Henle *in vivo* however showed that Na-acetate leaves the tubulus lumen by passive diffusion and not by active transport as Na-chloride and Na-bromide (unpublished data).

Thus, the feedback response is more likely to be initiated by a passive mechanism at the receptor site. The results of Wright and Persson (1974) that transepithelial hyperpolarization per se induced a feedback response (Wright and Persson, 1974) can also be in agreement with the latter explanation. Morphological support of the hypothesis of passive processes encountered into the feedback stimulation can be derived from the studies of Latta, who demonstrated that the morphology of the macula densa cells is different compared to the neighboring cells of the ascending limb and the early distal tubulus epithelium (Latta 1973). Furthermore, Beerwies, Shaboud and Rosen (1975) demonstrated the absence of transport enzymes such as Na-K ATPase in macula densa cells. They concluded that the postulated receptor function is not compatible with an active NaCl transport process. Recently in micropuncture studies by Navar and Bell (1977) these authors suggested that the perfusate osmolality may induce the feedback response.

Transmembrane or transepithelial potential difference changes in dependence of the tubular lumen strength could be one of possible passive steps initiating the tubuloglomerular feedback response. However the present studies cannot discriminate between the above-mentioned concepts. Direct experiments on surface macula densa cells and further biochemical analyses of single macula densa cells are needed to further clarify the mechanism.

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Electrochemical forces for chloride transport in the proximal tubules of the rat kidney

By

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Abstract

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The electrochemical forces for chloride transport in the proximal tubule of the rat kidney were studied using microelectrode techniques. Electrical transmembrane potentials were recorded in randomly punctured tubules with Lang-Gierard electrodes. Chloride activities in the lumenal, cellular and interstitial compartments were measured with ion selective micro-electrodes. Electrical potential measurements between cell to peritubular and lumen to interstitium were -72.1 ± 6 mV and $+0.5 \pm 1.4$ mV (mean \pm S.D.) respectively. The calculated chloride concentrations for lumen, cell and interstitium were 133.0 ± 10.3 mM, 8.5 ± 1.0 mM and 99.1 ± 2.2 mM (mean \pm S.D.) respectively. The net electrochemical forces, qualitatively offer passive chloride ion pathway through the tubular wall and chloride equilibrium over the horizontal membrane seems to exist.

Keywords: Chloride activity, electrical potentials, micro-electrodes, chloride transport, rat proximal tubule, rat.

Chloride and bicarbonate are the predominant anions of the glomerular filtrate and are mainly reabsorbed in the proximal tubule of the kidney (Windhager 1968). Walker *et al* (1961) reported a chloride concentration difference near unity across the proximal tubular wall in the early part, increasing to 1.4 in the middle and late parts. This concentration profile has been confirmed by Gottschalk (1963) and Danielson *et al* (1970). At various times, proximal tubular reabsorption of chloride has been regarded as a passive or active process, depending on the potential difference measurements obtained by different authors. Feltzer and Heyel (1966) first showed that no significant potential difference (PD) existed transcellularly which had earlier been reported to be 20 mV lumen negative (Windhager *et al* 1965).

Recently it has become obvious that the proximal tubular epithelia of the mammalian kidney under normal functional state, shows a potential difference profile along its length. Rabbit isolated proximal tubules *in vitro* showed a small negative potential which could be inhibited with ouabain (Burg and Orloff 1970, Kokko and Rector 1971). Using

a similar preparation, Kokko (1973) found a small positive potential unaffected by ouabain which was explained as secondary to chloride diffusion. Barratt *et al.* (1974) and Frerking and Gessner (1974) showed that the PD under free flow conditions in the very early part of the rat proximal tubule *in vivo* was slightly negative and changed to a small positive potential in the middle and late parts. These findings were recently confirmed by Seely and Chirito (1975).

According to the present knowledge regarding chloride concentration and PD profile in the proximal tubule, reabsorption of this ion is likely to be passive (Giebisch 1974), but the question remains as to whether the transport route is intra- or intercellular. The aim of this investigation was to study the electrochemical forces for chloride ion movement. The chloride activities of the proximal tubular lumen, the cell and the interstitium of the rat kidney were measured with chloride selective micro-electrodes of the liquid ion exchange type, used recently by Khuri (1974a) in renal research. The electrical potential difference between the compartments were measured with Ling-Gerhard electrodes. These recordings enabled calculation of the chemical transmembrane potential differences for chloride.

Methods

Sprague Dawley rats weighing 200–300 g. were used.

Anesthesia was induced with an intraperitoneal injection of Inactin (Chem. Fabr. Promonta, Hamburg, West Germany) in a dose of 120 mg/kg b.wt. Body temperature was maintained at 37°C with a servo-controlled heating table.

Animals were tracheostomized. The left jugular vein was cannulated and infusion of Ringer solution at a rate of ml/h and kg b.wt. started. A catheter was inserted to the urinary bladder for drainage, in order to reduce the influence of the micturition reflex. The left kidney was exposed via a flank incision and suspended in a Lucite cup. The kidney was embedded and immobilized in a solution of glycerol saline, except for a small part of the dorsal surface, which was superfused with warmed mineral oil. All the experiments were carried out in a Faraday cage. The proximal tubules were identified in UV light using a Ultrapak microscope (E. Leitz, GmbH, D330 Wetzlar, West Germany) and punctured randomly.

The transtubular potential difference (PD_{trans}) and the potential difference of the interstitium (subcapsular space) versus cell (PD_c) were recorded with Ling-Gerhard electrodes. The chloride activity of the interstitium (a_{Cl}^i) and the lumen (a_{Cl}^l) were measured with double-barrelled chloride selective electrodes. The intracellular chloride activity (a_{Cl}^c) was measured with a single-barrelled selective electrode with a reference electrode positioned in the interstitium. During the potential recordings, the infusion was stopped to prevent electrical interference.

The Ling-Gerhard electrode. The electrode (Fig. 1A) consisted of a 1.0 mm Pyrex glass capillary pulled to an outer diameter of about 10 μ m or 0.1–0.5 μ m (see results). The tip was filled with 1 M KCl from a catheter introduced into the rear end. The shaft of the electrode was connected to a slightly wider glass capillary filled with 1 M KCl, which had an Ag/AgCl wire glued to the inside with epoxy resin (UHU Plast, 3 M nutrient, UHU-Werk, H. u. M. Fischer 758, Buhl, Baden, West Germany). Electrodes with tip resistances of 5–150 Mohms were used. The asymmetry potential between two measuring electrodes was checked in Ringer solution and in the fluid of the subcapsular space before and after every recording. This potential was not allowed to be more than ± 5 mV and had to be stable otherwise the electrodes were rejected. The maximum deviation from the mean output of the recording device was ± 0.5 mV.

The chloride selectivity electrode. The single-barrelled electrode was of the same design as the Ling-Gerhard electrode, but the very tip contained a chloride selective liquid of the ion exchange type (Corning Scientific Instruments, Cat. No. 477315, England). Application of the selective liquid from the rear end into the tip was done through a catheter by applying a pressure more than 10 kg/cm². The length of the selective liquid column was about 0.5 mm. The capillary was then filled with 1 M KCl.

The double-barrelled electrode (Fig. 1B) was made of two glass capillaries. They were electrically heated kept close to each other, twisted 180° and then pulled to a tip with an outer diameter of less than 1 μ m. One tip was filled with chloride selective liquid and 1 M KCl as described and the other barrel with 1 M KCl only.

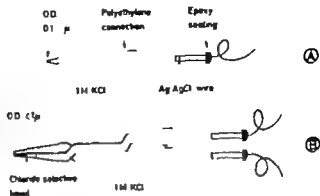


Fig. 1 The electrode design. A) The Ling-Gerhard electrode or the single-barrelled chloride selective electrode, which the very tip was filled with selective liquid. B) The double-barrelled chloride selective electrode.

Both the single and double electrodes were initially silicized (Denscoat, Beckman Instr. Inc., Fullerton, Cal., U.S.A.) to become hydrophobic before the application of the selective liquid. The chloride selective electrode was kept in air when not used. The electrodes were attached to an electrometer (Vibron Electro-meter II B, Electrosc. Instr. Ltd., Richmond, Surrey, England) and potentiometer recorder (Laboratory Recorder MKI, Yokogawa Electric Ltd., Tokyo, Japan).

The electrodes were calibrated with KCl standards ranging from 3 to 300 mM. The ionic strength of the standard fluid supposed to be the same as that for the luminal and interstitial fluids, and therefore the KCl standards corresponding to the intracellular recordings were corrected with the calculated activity factor of 0.71 (Oster 1959) for an ionic strength of 130 mM. In the following the concentration values were obtained by dividing the activity values with the factor 0.71. The sensitivity mV/pCl, at 37°C for all the single electrodes ranged from 57 to 60, and for all the double electrodes from 49 to 54. The response time was less than 1 s and the tip resistance around 10^9 ohms for both electrode systems.

In measuring the chemical potential differences, Nernst equation was used.

$$E_{Cl} = -\frac{RT}{zF} \ln \frac{a_2}{a_1}$$

where E_{Cl} the difference potential, R , T and F have their usual meaning; a_2 and a_1 express the chloride activity on the respective sides of the membrane.

Results

Measurements were subdivided into 4 series. The first and second series dealt with the PD_{lum} and PD_{int} , the third with the chloride concentration of the interstitium (Cl^-_i) and luminal lumen (Cl^-_l) and the fourth with that of the cell (Cl^-_c).

PD_{lum} . The proximal transtubular potential difference was measured with the Ling-Gerhard electrode and the results are shown as a frequency histogram in Fig. 2. The PD_{lum} is 0.5 ± 1.4 mV (mean \pm S.D. $n = 100$) and significantly different from zero ($P < 0.001$).

PD_{int} . When the outer diameter of the tip of the Ling-Gerhard electrode was about 10 μm, the potential difference of the interstitium versus cell was generally transient and showed large amplitude scattering. When the diameter was minimized to 0.1–0.5 μm, the potentials became stable and could be recorded for up to 10 min, or until the recording was interrupted for the next puncture (Fig. 3). The distribution of the potential differences observed is presented in Fig. 4. The PD_{int} from the second series was -72.1 ± 2.6 mV (mean \pm S.D. $n = 53$).

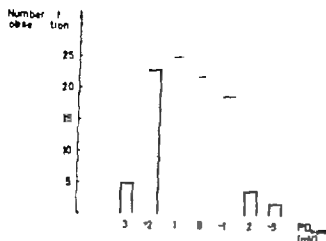


Fig. 2

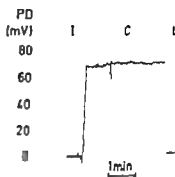


Fig. 3

Fig. 2. The histogram shows the transcellular potential differences (PD_{trans}) measured in the proximal tubule.

Fig. 3. A single recording of the potential measurements with Ling-Gerhard electrodes. I = interstitium, C = tubular cell, L = tubular lumen.

$(Cl^-)_i$ The recordings of the chloride activity in the interstitium were undertaken with the double-barrelled type of electrode. The chloride concentration was 99.1 ± 3.2 mM (mean \pm S.D. $n=9$).

$(Cl^-)_c$ The chloride activity of the tubular lumen was also measured using the double-barrelled electrode. The recorded value corresponds to a concentration value of 133.0 ± 10 mM (mean \pm S.D. $n=11$).

$(Cl^-)_i$ The intracellular chloride activity was measured with a single-barrelled chloride electrode. The potentials recorded were, thus, the sum of the potential for the a_{Cl^-} and the PD_{i-c} (Fig. 5). The electrodes showed stable sensitivities, mV/pCl while a slight parallel shift of the calibration line could occur. Therefore, the a_{Cl^-} potential was always referred to the actual potential for the $(Cl^-)_i$ of 99.1 mM (see above). The calculated intracellular chloride concentration (Fig. 6) was then 8.5 ± 1.0 mM (mean \pm S.D. $n=60$).

The chloride chemical potential differences $(E_{Cl}) \pm$ S.E. across the tubular wall, luminal membrane and peritubular membrane were calculated to be 7.9 ± 2.2 , 73.5 ± 3.8 and 65.6 ± 3.3 mV respectively. By combining the membrane potential PD and E_{Cl} the net electro-

Number of
observations

10

5



Fig. 4. The histogram shows the potential differences between the interstitium and the tubular cell (PD_{1-c}).

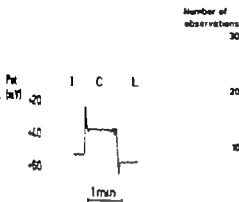


Fig. 5

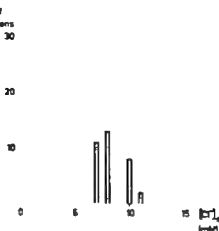


Fig. 6

Fig. 5 A single recording of the chloride activity potential measurement with the single-barrelled chloride selective electrode.

Fig. 6 The integrator shows the calculated intracellular chloride concentration, $[Cl^-]_i$, measured with the single-barrelled chloride selective electrode.

luminal driving force ($NetE_{Cl}$) \pm S.E. over the tubular wall was estimated to be 7.4 ± 2.6 mV which was significantly different from zero ($p < 0.01$). The $NetE_{Cl}$ for the luminal and peritubular membranes were 0.9 ± 4.8 and 6.5 ± 4.2 mV respectively and not significantly different from zero. The estimations of S.E. were done according to Hald (1952).

Discussion

Many technical problems arise in attempting to measure electrical potentials and ion activities in small structures. The difficulties of localizing the electrode tip in the tubular lumen and in microtubules, which has been discussed in the literature by Boulpaep (1972) and Frömter (1972), were overcome using a high resolution microscope. In this investigation, when measuring the transtubular PD the electrode tip was placed in the tubular lumen and then withdrawn inside the lumen in the tubular direction about one luminal diameter whereby the recorded potential was constant. Attachment of the tip to the brush-border was easily observed in the microscope and the potential obtained amounted to about -20 mV which may be responsible for the disparities among the recorded values obtained by different authors.

The results of the PD_{trans} for the rat kidney as recorded by random puncturing of the proximal tubule under free-flow conditions, are in agreement with recently measured intracellular potentials (Barratt *et al.* 1974, Frömter *et al.* 1974, Khuri *et al.* 1974 b, Seely *et al.* 1975). The scattering of the potential values seems to be explained by the potential profile along the proximal tubule (Barratt *et al.* 1974, Frömter *et al.* 1974, Seely

et al 1975) the early part is slightly negative while the middle and late parts become slightly positive. The measured values of the PD_{1-0} gave a potential distribution from 0 to 80 mV (cell negative). This pattern has been reported previously by Frömter *et al* (1966) and Sohtell and Ulfendahl (1973). The potential scatter seems to be explained partly by membrane damages and partly by the likelihood that the electrode tip was located in the cellular membrane. The latter assumption is very plausible in view of the numerous lateral foldings of the proximal tubular cell. When the size of the tips ranged from 0.1 to 0.5 μ m the pattern of the PD_{1-0} recordings altered. When advancing the electrode tip into the cell the potential changed abruptly and was in most cases stable for minutes. The mean potential of -72.1 mV is in agreement with the values of -74 mV reported by Frömter *et al* (1966) and -68.5 mV obtained by Khuri *et al* (1974 b).

The interstitial chloride concentration (Cl^-), 99.1 ± 3.2 mM (mean \pm S.D.) was found to be of the same order in all experiments and the deviation was small. The chloride concentration potential of the interstitium was therefore used as a control of the stability of the chloride electrode. The random luminal concentration varied between 115 and 150 mM which is in accordance with earlier findings (Walker *et al*, 1941; Gottschalk 1966; Danielson *et al* 1970).

The double-barrelled electrode was used initially for a_{Cl^-} determinations, but stable recordings were not generally obtained. This was almost certainly due to leakage between the cell membrane and the electrode, as the circumference of the tip was not circular but rather "eight"-shaped. For this reason the single-barrelled chloride electrode was used to measure a_{Cl^-} . To date no method exists for measuring different ion concentrations in the cytoplasmic fluid of the proximal tubular cells, a value which is necessary when calculating the ionic strength. The calculated activity factor (0.71) is therefore regarded as a reasonable approximated value valid for all renal structures.

Nernst's equation, which was used for the calculation of the electrochemical potential, can only be used under equilibrium conditions. A maximum potential gradient predicted for passive diffusion through the cell can then be estimated according to the equation. Seen on the proximal tubule as a unit when randomly punctured the calculated electrochemical forces across the tubular wall which were found significantly different from zero, give a passive driving force for chloride movement. This movement is also favoured by a high chloride permeability (Radtke *et al* 1971; Frömter *et al* 1971).

Much discussion has centered around the relative importance of para- and transcellular ion transport in various epithelia e.g. gall bladder (Diamond 1962). In the proximal tubule the electrochemical significance of the intercellular space for chloride transport with its tight junction near the luminal membrane, has been reviewed by Gleibach *et al* (1973). This pathway seems to be responsible for a low tubular electrical resistance, a high ion and water permeability, a low fixed charge density and a marked lack of ion restrictivity. These properties and the electrochemical driving forces across the tubular wall for chloride ions found in this investigation make it plausible to assume a paracellular pathway for chloride.

The chloride ion transport over the luminal membrane seems to be in an equilibrium state, as Net E_{Cl^-} was found small and could not be judged to be significantly different from zero as randomly measured in this investigation. This, however, does not exclude partial net

chloride movement over the luminal membranes along the proximal tubule according to the literature named luminal chloride concentration profile.

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Blood velocity in human arteries measured by a bidirectional ultrasonic doppler flowmeter

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Abstract

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Blood velocities in 12 arteries were recorded by an ultrasonic doppler flowmeter in 11 young adults. The major types of velocity patterns existed at rest. In certain arteries (the common carotid, the external carotid, the superficial temporal and the proper palmar digital arteries) flow was towards the periphery throughout the entire pulse cycle. Other arteries (the common femoral, the popliteal, the posterior tibial and the pedal artery) exhibited retrograde flow in part of the pulse cycle. In each individual spontaneous variations between these two velocity patterns was observed in the subclavian, the axillary, the brachial and the radial artery. The velocity pattern of each artery is described, and absolute blood velocities at recognizable instances during the pulse cycle are given. The influence of peripheral resistance on the velocity pattern was investigated by reactive hyperaemia of the femoral artery. We find that not only is there an upward displacement of the resting femoral curve relative to the line of zero, but the shape of the velocity pattern is also changed. Our conclusion is that peripheral resistance is of major importance not only for the mean velocity but also for the shape of the velocity pattern in the artery.

Instantaneous blood flow patterns have been recorded peroperatively for some time by the use of electromagnetic flowmeters (e.g. Schenk *et al.* 1960, Gault *et al.* 1966, Lauridsen 1968, Dedichen and Kordt 1974). These measurements suffer certain limitations, most notably the unknown influence of anaesthetics, drugs and surgical procedure and the restricted number of vessels available for examination in each patient. The bidirectional ultrasonic doppler flowmeter is used transcutaneously and permits blood velocity recording from a number of arteries without surgery. Such transcutaneously performed measurements do not interfere with physiological conditions. The disadvantages are that the angle between the sound beam and the flow direction has some influence on velocity determination and that volume flow is not yet directly accessible.

Before changes in the velocity patterns with age and under pathological conditions are investigated, a study of young normal adults is needed. Nimura *et al.* (1974) give forward and retrograde peak velocities in 24 healthy individuals, age 22-38 years, for 4 arteries (the

aorta, the brachial, the radial and the common femoral), and comment on the parts of two others (the subclavian and the pedal artery). We find their velocities surprisingly large and doubt whether volume flow if calculated from the mean velocity during pulse cycle and an assumed arterial diameter would be within the range of possible flow rates. Our primary aim was, therefore, to repeat these investigations and to establish reasonable reference velocity values at certain recognizable instances during the pulse cycle in young normal individuals.

Kamra *et al.* (1974) describe distinct differences between the velocity patterns of the aorta, the brachial and the femoral arteries. We have included more arteries in our study in as if all these arteries could be classified in one of Nixura's groups or if other patterns could be observed.

Further Nixura *et al.* (1974) observed that the reverse flow phase was propagated from proximal to distal. They suggest that "the reverse flow phase did not result from a rebound from the periphery" but largely from the nature of the vessel wall, *i.e.* elasticity. To investigate the contribution peripheral resistance as opposed to elasticity and other qualities of the arterial wall might give to the velocity pattern, we recorded from the common femoral artery at rest and during reactive hyperaemia.

Materials and methods

Measure of blood flow velocity were performed on 11 individuals (8 male, 3 female, mean age 24 years range 23-71), none of whom were known to have any cardiovascular disease. All measurements were made with the subject in the supine position. 12 arteries were studied at rest, and the common femoral velocity pattern was recorded after tourniquet occlusion. An inflatable cuff was applied to the thigh with its upper edge 10-15 cm below the hip joint. Control registrations were made before inflation. The cuff was inflated to 200 mmHg for 2 min, which would give 93% hyperaemia response compared to 5 min occlusion (DeLichs and Mylre 1975). All recordings were taken within 15 s after release of pressure. In this way we obtained low resistance flow patterns without an increase in heart rate or blood pressure.

The Doppler flow meter, which can discriminate between forward and retrograde blood velocities ("bidirectional") was built in the department. Parts of the operating technique have been described elsewhere (Srinivas 1979). Two sound frequencies are available, 2 MHz and 6 MHz. The emitted beam can be pulsed continuously. In this investigation the 6 MHz frequency was used. The bulk of the carotid and the femoral segments were obtained with pulsed beams, while recordings from other arteries were achieved by continuous scan. A gel applied to the skin ensured acoustic contact. The probe angle was approx. 45°. Corrections of velocity were made for the apparent angle between the ultrasonic beam and the mean direction of flow.

Following frequency analysis, the signals appeared on a storage display unit. An original recording is shown in Fig. 1 (photo). All velocities present within 25 ms sampling interval occurred as vertical spikes. A long column would reflect the simultaneous presence of a large range of velocities within the vessel. This is seen in small arteries where the beam would include the entire cross section of the vessel, but could be observed in larger arteries when the sampling site was relatively close to the wall. Doppler shift is less than 200 Hz (velocities smaller than 0.033 m/s) would not be detected. By switch the acoustic scale could be doubled or tripled in height. The display was photographed with an instant developing polaroid film. Maximum linear blood velocity in each sampling was then plotted as a function of time.

We have calculated approximate volume flows based on reported mean diameters in x-ray literature and rough estimate of the mean velocity of each artery. The following equations have been used

$$max = \frac{\Delta f}{2f \cos \theta} \quad (1)$$

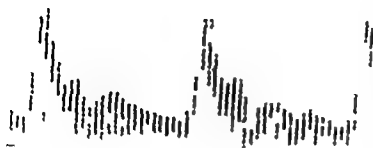


Fig. 1. An original oscilloscope recording from the common carotid artery as it appears on the size display unit. The display has been photographed with instant developing polaroid film. Abscissa: time, ordinate: blood velocity. Height of columns is proportional to blood velocity. The velocity curves presented in this paper present the maximum blood velocity occurring at any instant time.

$$v_{\text{mean}} = \frac{v_{\text{max}}}{2}$$

$$v_{\text{mean}} = \frac{1}{T} \int_0^T v_{\text{peak}} dt$$

$$Q = \pi r^2 v_{\text{mean}}$$

where Q = volume flow

r = radius

v_{max} = instantaneous maximum velocity across the lumen

v_{peak} = instantaneous mean velocity across the lumen

v_{mean} = time average of the instantaneous mean velocity

c = sound velocity

T = duration of the cardiac cycle

Δf = doppler frequency shift

f = emitting frequency

θ = angle between the sound beam and the flow distribution

By using equation (2) with a assumed parabolic velocity profile in the artery. This assumption is also used by Nimura *et al.* (1974) and most other authors, but is questionable, and will be examined in detail in a following paper.

Results

Blood flow velocity curves

Two major types of velocity patterns were noted to exist. Certain arteries showed "forward" flow (that is flow towards the periphery) throughout the pulse cycle. This pattern was observed in the common carotid, the external carotid, the superficial temporal and the proper palmar digital arteries.

Other arteries exhibited retrograde flow in part of the pulse cycle. A retrograde flow component was always present in registrations of the common femoral, the popliteal, the posterior tibial and the pedal arteries.

In addition there were some arteries which showed a non-steady flow pattern. Repetitive registrations of the same individual displayed both of the two patterns mentioned above, though not at the same time. A non-steady flow pattern was demonstrated in the subclavian, the axillary, the brachial and the radial arteries.

Time spent in arterial "systole" was 225–300 ms in all registrations except for those of the brachial and radial arteries when retrograde flow was present resulting in a "systolic" time of 125–175 ms. When heart frequency was increased by a factor of 1.6 (registrations

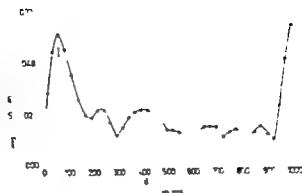


Fig. 2. An example of the common carotid velocity pattern. Peak velocity (a) occurs early in arterial "pulse" (b to c). The slope of steadily decreasing velocities following the peak is interrupted by small flow (200 ms). The "diastolic" flow cycle (c to) is composed of well-defined peak followed by oscillating flow velocity.

less shortly after running), "systolic" time remained unchanged or shortened only by 25 ms.

The common carotid artery. Recordings were done at the level of the cricoid cartilage. The most striking feature of the registrations is that flow is always "forward" that is towards the periphery. Also, flow never falls to zero. The flow cycle can be divided into a "systolic" and a "diastolic" part (see Fig. 2). Peak "systolic" flow velocity (b) was reached in 50-75 ms. During the remaining part of the "systole" flow velocity fell steadily except for a notch half-way from b to c. Diastolic flow cycle started with a well-defined peak which was followed by oscillating flow varying both in amplitude and regularity of oscillations. Figures for flow velocity and time relations are presented in Table I.

The external carotid, superficial temporal and proper palmar digital arteries. Measurements of blood velocities in the external carotid artery were taken at the level of the mandibular angle, while those of the superficial temporal artery refer to flow at the level of the external meatus of the ear. The velocity patterns were very similar to that of the common carotid artery. For details we refer to Table I.

Measurements of the proper palmar digital arteries were made close to the distal interphalangeal joint. The flow pattern differed only slightly from the carotid flow pattern. The slope of decreasing maximum velocity following the "systolic" peak was less steep and rarely was there a notch present. The point of end "systole" was less clear-cut, but was followed by a rounded peak as in the common carotid. Peak velocity averaged 0.19 m/s, velocity at end diastole measured 0.07 m/s. Time relations matched those reported in Table I.

The common femoral artery. Measurements were performed immediately distal to the epiphyseal ligament. All registrations showed forward flow wave with a sharp increase before and a sharp decrease after a high peak (Fig. 3). This was invariably followed by a period of backflow and a second forward flow wave of less magnitude before the flow fell to zero. The part of arterial diastole without detectable flow lasted for 150-475 ms. Velocities and time relations are given in Table I.

TABLE I Blood flow velocities and time intervals of the flow cycle in arteries. All figures are based on registrations from 11 individuals, and are median velocity values. Velocity ranges are given in brackets. Where a spontaneous variation in the velocity pattern occurred, the presence of retrograde flow is marked b, while the continuous forward flow pattern is marked f.

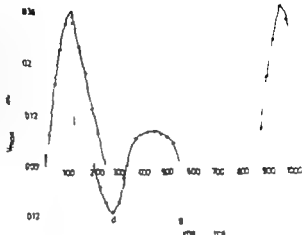
Artery	"Systolic rise time (a to b in ms)	Backflow duration (ms)	Peak systolic velocity (m/s)	End systolic velocity (m/s)	2nd forward peak velocity (m/s)	End diastolic velocity (m/s)
Common carotid	50-75		0.67 (0.40-0.84)	0.13 (0.10-0.26)	0.30 (0.16-0.36)	0.14 (0.04-0.27)
External carotid	50-75		0.48 (0.25-0.65)	0.15 (0.08-0.21)	0.25 (0.11-0.32)	0.12 (0.03-0.17)
Superficial temporal	50-75		0.48 (0.34-0.66)	0.11 (0.08-0.17)	0.23 (0.16-0.29)	0.07 (0.01-0.14)
Common femoral	75-125	125-200	0.38 (0.27-0.47)	0.09 (0.05-0.14)	0.08 (0.06-0.12)	0
Popliteal	50-125	100-250	0.8 (0.15-0.48)	0.10 (0.04-0.19)	0.06 (0.04-0.15)	0
Posterior tibial	50-100	100-200	0.24 (0.19-0.40)	0.07 (0.03-0.13)	0.06 (0.03-0.09)	0
Dorsal artery of the foot	50-100	100-200	0.26 (0.20-0.51)	0.08 (0.05-0.11)	0.05 (0.03-0.16)	0
Subclavian ^b	50-100	50-200	0.65 (0.43-0.78)	0.09 (0.04-0.16)	0.14 (0.06-0.1)	0
Axillary ^b	75-100	50-200	0.31 (0.20-0.45)	0.05 (0.04-0.09)	0.08 (0.05-0.15)	0
Axillary ^f	75		0.33 (0.27-0.45)	0.06 (0.03-0.12)	0.11 (0.10-0.20)	0.05 (0.03-0.09)
Brachial ^b	50-75	75-150	0.38 (0.35-0.76)	0.07 (0.05-0.15)	0.10 (0.06-0.26)	0
Brachial ^f	75		0.54 (0.34-0.73)	0.07 (0.03-0.16)	0.19 (0.11-0.31)	0.06 (0.00-0.12)
Radial ^b	50-75	50-125	0.24 (0.19-0.31)	0.04 (0.03-0.06)	0.05 (0.05-0.07)	0
Radial ^f	50-100		0.31 (0.24-0.52)	0.06 (0.01-0.11)	0.12 (0.08-0.20)	0.05 (0.00-0.19)

The popliteal posterior tibial and pedal arteries The popliteal artery was studied at the knee joint, the posterior tibial at the level of the medial malleolus and the dorsal artery of the foot at a level proximal to the arcuate artery. All registrations showed a pattern similar to that of the common femoral artery. For details see Table I.

The subclavian artery Recordings were made in the supraclavicular triangle. The probe angle was estimated to 60° based on anatomical considerations. All registrations except one showed backflow in part of the pulse cycle.

A subclavian artery registration is presented in Fig. 4. A notch halfway from b to d (as described in the common carotid) often occurred. More than half of the registrations showed a period (25-250 ms) of simultaneous forward and retrograde flow. This was observed in other arteries as well but was never as striking as in the subclavian recordings. A second

Fig. 3 is a sample of the common carotid velocity curve. The "systolic" plot (a to d) is composed of forward flow wave (a to c) through left port (b) and the development of retrograde flow (a to d) during the period of simultaneous forward and retrograde waves (c to d). A second forward flow wave (e to g) is present, followed by a period of no velocity due to flow (g to h). Maximum "systolic" forward flow is at h.



use of backflow occupying 125-200 ms, peak velocity approximately 0.05 m/s, was usually present. For further information see Table I.

Axillary brachial and radial arteries. Blood velocities of the axillary artery was measured in the axilla. 4 registrations showed an uninterrupted forward flow with a carotid type of pattern. The remaining recordings resembled those of the subclavian artery. A second wave of backflow was present in one registration.

The brachial artery recordings were performed at the elbow joint and the radial ones at the wrist. All subjects demonstrated both a flow pattern like that of the femoral artery and a flow pattern resembling the carotid velocity curve. This change in flow pattern occurred without any change in body position or environmental temperature during the period of measurements. When backflow was present, the second forward flow wave was split into two separate peaks in 4 subjects.

Numeric descriptions of all arteries are given in Table I.

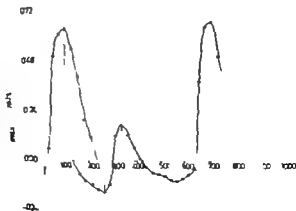


Fig. 4 Plot of recording from the subclavian artery when backflow was present. The velocity pattern resembles that of the common femoral artery in more than half the subjects, however there was a second wave of backflow (g to h) of varying magnitude and duration. The letters used correspond to those in Fig. 3.

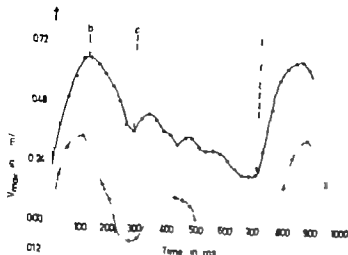


Fig. 5. The velocity patterns of common femoral artery at rest (stippled) and following a 2 minutes tourniquet occlusion (drawn) from individual are plotted in the diagram for comparison. During reactive hyperemia all flow is towards the periphery and velocity are high. The "systolic time" (c to) is unchanged while the stroke (c to) is slightly shorter. End "systolic" velocity (c) higher than end diastolic contrary to the findings at rest.

Reactive hyperemia (The common femoral artery)

Registrations taken within 15 s after release of a 2 minutes tourniquet occlusion show marked differences from resting femoral flow pattern (Fig. 5). All flow was towards periphery and the velocity never fell to zero. The high peak of the "systolic" forward flow at rest persisted but compared to resting pattern there was a less sharp decrease in velocity after the peak was reached. An end "systolic" point indicated a "systolic time" of 225-300 ms. This was followed by a second smaller peak.

Median blood flow velocity was at the "systolic" peak 0.62 m/s, at end "systolic" 0.25 m/s, and at end "diastole" 0.19 m/s. Note that the end "systolic" velocities are higher than the end "diastolic" values, contrary to the findings of the resting flow pattern.

Discussion

Few reports on instantaneous blood flow patterns are available, and in most cases the registrations are obtained peroperatively by an electromagnetic flowmeter. We have found common femoral velocity curves corresponding closely to the electromagnetically recorded flow pattern reported by Dedichen and Kordt (1974). In the brachial artery we observed a spontaneous variation in velocity pattern at rest as did Gault *et al.* (1966).

Nimura *et al.* (1974) have recorded velocity curves in young healthy adults (age 22-33 years) by an ultrasonic doppler flowmeter. The common carotid, the subclavian, the brachial, the radial, the femoral and the pedal artery were investigated. Generally there is a close correspondence between the shape of their velocity curves and ours. We do not agree, however, that blood velocity is low in the pedal artery and all of our recordings of this artery exhibited backflow. The most striking difference between the ultrasonic recordings of Nimura *et al.* and our registrations is to be found in peak velocity values. Their figures are 2-3 times as high as our findings. We have already pointed out that this will give unreasonably high volume flow values.

Introducing reactive hyperemia by a tourniquet occlusion is a way of selectively lowering the peripheral resistance without increasing the heart rate or the blood pressure. Nor are

TABLE II. Estimated and reference volume flow

Artery	Diameter in cm	Mean velocity in m/s (= mean)	Calculated volume flow in ml/min (Q)	Electromagnetic volume flow in ml/min
Common carotid	0.7 ^a	0.21	485	250-450 (1), 500 (2)
External carotid	0.4 ^a	0.19	143	100-200 (1), 150 (2)
Superficial temporal	0.3	0.16	68	
Common femoral	0.85 ^a	0.07	238	228 (3), 239 (4), 320 (5)
Femoral	0.7 ^a	0.05	116	
Popliteal	0.32 ^a	0.04	19	
Pedal	0.25	0.06	18	
Subclavian	0.9 ^a	0.06	229	250 (6)
Axillary	0.7 ^a	0.06 ^b /0.07 ^c	138 ^b /162 ^c	160 (7)
Brachial	0.55 ^a	0.06/0.12 ^c	86 ^b /173 ^c	23-107 (8), 7-169 (9)
Radial	0.25	0.04 ^b /0.10 ^c	23 ^b /58 ^c	
Proper palmar digital	0.1	0.12	5	

References for arterial diameter inserted are taken from Latta (1974). The figures show reference values for own estimates of vessel diameter.

Figures marked ^b refer to backflow pattern, while those marked ^c refer to continuous forward flow. All calculations are based on an estimated angle of 45° between the sound bases and the main direction of flow, except those of the subclavian artery have approximate angle according to anatomical considerations have been 60°.

References for volume flow measured by an electromagnetic flow meter have been: 1 Nordnes, H. personal communication, 2 and Krog (1962), 3 Dedichen and Myhre (1975), 4 Våltræen (1975), 5 Lundness (1972), 6 Dedichen, H., personal communication, 7 Gault *et al.* (1966), 8 Langhans.

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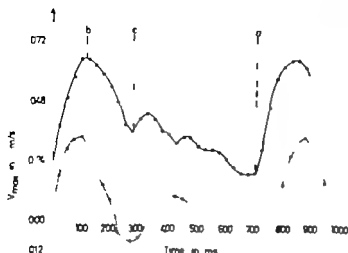


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References for arterial diameter marked ^a are taken from Luzzo (1974). The figures without reference represent our own estimates of vessel diameter.

Figures marked ^b refer to backflow pattern, while those marked ^c refer to continuous forward flow. All calculations are based on an estimated angle of 45° between the sound beam and the main direction of flow, except those of the subclavian artery where approximate angle according to anatomical considerations was less, 60°.

References for volume flow measured by an electromagnetic flowmeter have been: 1. Nordnes, H., personal communication, 2. Kristensen and Krog (1962), 3. Dedichen and Myrnes (1975), 4. Vilmann (1975), 5. Lenné (1960), 6. Midtved (1972), 7. Dedichen, H., personal communication, 8. Gault *et al.* (1966), 9. Langbein *et al.* (1974).

properties of the arterial wall changed. If peripheral resistance is of minor importance for the time pattern of the instantaneous velocities, only an upward displacement of the resulting femoral curve relative to the line of zero (corresponding to increased mean flow) would be expected in registrations during hyperaemia. However the registrations showed forward flow through the entire pulse cycle, and, most notably end "systolic" velocities are higher than end "diastolic" ones. The pattern resembles that of the common carotid. Failure to demonstrate a fully established carotid pattern may be due to a remaining resistance in thigh microcirculation. Our conclusion is that peripheral resistance is of major importance not only for the mean velocities, but also for the shape of the velocity pattern in the artery.

In some of the arteries where backflow appeared our recordings showed a period (25-50 ms) in which both forward and retrograde blood flow was simultaneously present in the artery. This phenomenon occurred in "systole" or in diastole or in both, and was also observed by Nimura *et al.* (1974). We believe this is a visible image of the theoretical considerations by Wormersley and McDonald (cited in McDonald 1974). According to their theory the fluid laminae nearest the wall reverse earlier than those closer to the axis of the tube. Peak velocities, however, are caused by the laminae midstream.

Estimated volume flows are given in Table II. Our calculations are based on reported mean diameters in x-ray literature and on the calculated mean velocity of all recordings from each artery. A reference to electromagnetic results is given where these are available.

A possible underestimation of the popliteal volume flow must be considered as popliteal blood velocities were recorded with the subjects on their backs and with a knee flexion. Reduced flow in the femoral artery when a hip joint flexion is present has been reported (Hall 1969).

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Different effects of the ionophore A 23187 and D-glucose on $^{45}\text{Ca}^{++}$ fluxes in isolated islets of ob/ob-mice

By

JOSÉ TAMARIT RODRIGUEZ

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Abstract

TAMARIT-RODRIGUEZ, J. Different effects of the ionophore A-23187 and D-glucose on $^{45}\text{Ca}^{++}$ fluxes in isolated islets of ob/ob-mice. Acta physiol. scand. 1978. 103. 379-383.

Fluxes of $^{45}\text{Ca}^{++}$ were studied in β -cell rich islets of non-obese ob/ob-mice, using LaCl_3 to wash out extracellular and specifically bound $^{45}\text{Ca}^{++}$. The ionophore A 23187 (10 μM) increased the $^{45}\text{Ca}^{++}$ uptake in islets both in 3 and 20 mM D-glucose, the effect being more pronounced after 10 min than after 120 min of incubation. In incubations for 120 min, 20 mM D-glucose induced a higher uptake of $^{45}\text{Ca}^{++}$ than did A-23187. The ionophore enhanced the unidirectional efflux of $^{45}\text{Ca}^{++}$ from prelabeled islets. Pretreatment of islets with 20 mM D-glucose in non-radioactive medium inhibited the subsequent D-glucose-induced $^{45}\text{Ca}^{++}$ uptake. Similar pretreatment with A-23187 increased the subsequent ionophore-induced $^{45}\text{Ca}^{++}$ uptake. The results suggest that A 23187 acts by catalyzing Ca^{++} fluxes across the β -cell plasma membrane. The different effects of D-glucose and A-23187 on $^{45}\text{Ca}^{++}$ fluxes suggest that the two agents act through different mechanisms in the β -cells.

Insulin stimulation of insulin release is generally assumed to be mediated by Ca^{++} ; several authors have investigated the effects of the Ca^{++} transporting ionophore A 23187 on insulin release (Ashby and Speake 1975, Charles *et al.* 1975, Hellman, 1975, Kari *et al.* 1975, Wolfheim *et al.* 1975, Conway *et al.* 1976, Somers *et al.* 1976). The rationale for these experiments has been the assumption that A 23187 affects Ca^{++} fluxes in the β -cells. However, in spite of the fact that data on ionophore effects on insulin release may be of great importance for the interpretation of Ca^{++} action in secretion, only little is known about ionophore effects on β -cell fluxes of Ca^{++} . The aim of the present work was therefore to investigate the effects of A 23187 on $^{45}\text{Ca}^{++}$ uptake and efflux in isolated islets from ob/ob-mice. In particular it was decided to compare the effects of ionophore with those of D-glucose, which is thought to induce secretion by affecting Ca^{++} fluxes.

Materials and methods

A 23187 was a gift from E. L. Lilly & Co. Indianapolis, Ind., U.S.A. $^{45}\text{CaCl}_2$ was obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and was added in trace amounts to incubation media giving

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specific radioactivity of about 7.8 Ci/mole Dimethylsulfoxide (DMSO) was supplied by Mallinckrodt Chemical Works St. Louis, Mo. U.S.A. All other conventional reagents were of analytical grade.

Adult non-inbred *ob/ob*-mice of the Umeå colony starved overnight were used for all expts. Pancreatic islets were isolated from the pancreas by free-hand microdissection (Hellstrom 1964, Coma et al. 1977) in basal medium at 4°C. The basal medium used in microdissection and subsequent incubations contained in mM: D-glucose 3.0, 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), 5.0; Na⁺ 139.0; K⁺ 4.7; Ca²⁺ 2.6; Mg²⁺ 1.2; Cl⁻ 151.2 as well as Cl⁻ added as HCl to give a final pH of 7.4. A 23187 (10 µM) added to the medium from a 10⁶ times more concentrated solution in DMSO, the concentration of DMSO in the medium was 0.1% (v/v). 0.1% DMSO was routinely included in all control incubation media.

⁴⁵Ca²⁺ fluxes were studied as previously described (Hellman et al. 1976), by measuring the content of ⁴⁵Ca²⁺ after washing the islets for 60 min in non-radioactive basal medium containing 2 mM LaCl₃. Tracer radioactivity in the islets was expressed in terms of nmol Ca²⁺ with the same specific labelling as in the medium. Samples of labelled medium (5/1) were used as external standards in the counting procedure.

Results

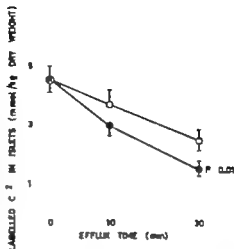
Previous work showed that D-glucose increases the uptake of ⁴⁵Ca²⁺ in the La³⁺-nonreplaceable (intracellular) pool in pancreatic islets from *ob/ob*-mice (Hellman et al. 1976). In accordance with this finding, Table I shows that islets incubated with ⁴⁵Ca²⁺ for 10 or 120 min in the presence of 20 mM D-glucose contained more ⁴⁵Ca²⁺ as compared to islets incubated with 3 mM D-glucose. The addition of 10 µM A 23187 markedly increased the ⁴⁵Ca²⁺ uptake at either 3 or 20 mM D-glucose.

The unidirectional efflux of ⁴⁵Ca²⁺ from pancreatic islets was measured by preloading islets with ⁴⁵Ca²⁺ at 3 mM D-glucose for 120 min followed by incubation in non-radioactive media. Fig. 1 shows that 10 µM A 23187 when included in the non-radioactive medium increased the efflux of ⁴⁵Ca²⁺ after 10 and 30 min of efflux. The same results were obtained when the ionophore was included during preloading of islets with ⁴⁵Ca²⁺ but excluded from the non-radioactive medium (not shown).

TABLE I Effects of A 23187 on ⁴⁵Ca²⁺-uptake. Batches of 5 islets were preincubated in basal medium (3 mM D-glucose) at 37°C for 30 min and then incubated for 10 or 120 min in the same medium supplemented with trace amounts of ⁴⁵Ca²⁺ (7.8 Ci/mol) and the additives indicated in the table. After incubation with ⁴⁵Ca²⁺ islets were transferred to a glucose- and calcium-free basal medium containing 2 mM LaCl₃ and incubated for 1 h at 37°C. Values are mean values ± S.E. for the number of experiments given in parentheses. * P < 0.05, ** P < 0.01, *** P < 0.005, § P < 0.001 when compared to 6.93 ± 0.78, 11 P < 0.05 when compared to 9.26 ± 0.96.

Additives	Uptake of labelled calcium (nmol/kg dry wt. of islets)	Differences from control
<i>10 min of incubation</i>		
3 mM D-glucose (control)	1.90 ± 0.33 (5)	—
3 mM D-glucose + 10 µM A 23187	5.93 ± 0.78 (5)	4.03 ± 0.74
20 mM D-glucose	2.74 ± 0.36 (5)	0.84 ± 0.23
20 mM D-glucose + 10 µM A 23187	6.02 ± 0.37 (5)	4.11 ± 0.51
<i>120 min of incubation</i>		
3 mM D-glucose (control)	3.06 ± 0.32 (6)	—
3 mM D-glucose + 10 µM A 23187	6.93 ± 0.78 (6)	1.87 ± 0.69
20 mM D-glucose	9.26 ± 0.96 (6) §	4.20 ± 0.81
20 mM D-glucose + 10 µM A 23187	11.62 ± 1.26 (6) 11	6.56 ± 1.11

Fig. 1 Effect of A-23187 on efflux of ⁴⁵Ca²⁺ from pancreatic islets. Batched of 5 islets were, after preincubation in non-radioactive basal medium (3 mM D-glucose, incubated for 120 min in the same medium supplemented with trace amounts of ⁴⁵Ca²⁺ (2.0 Ci/mol). The islets were then transferred to non-radioactive media without ionophore (○) or with 10 μM A-23187 (●) and incubated for the periods indicated on the figure, followed by incubation with LiCl₂. Table I Values are mean values ± S.E. for 3 separate experiments.



D-glucose has been suggested to stimulate the uptake of ⁴⁵Ca²⁺ in an intracellular calcium experiment with a low turn-over rate and high affinity for the ion (Heikman *et al.* 1976). The idea was further tested in the present study by pretreating islets with 20 mM D-glucose for various periods of time in non-radioactive calcium containing media and subsequently measuring the ⁴⁵Ca²⁺ uptake. Table II shows that islets preincubated with 20 mM D-glucose for 10 to 60 min took up significantly less ⁴⁵Ca²⁺ in response to 20 mM D-glucose than islets preincubated only at 3 mM D-glucose. Preincubation of islets with 10 μM A-23187 in non-radioactive media for 30 or 60 min did not decrease the subsequent ionophore induced ⁴⁵Ca²⁺ uptake but significantly increase it (Table III).

Table II Effects of preincubation with 20 mM D-glucose on the subsequent glucose-stimulated ⁴⁵Ca²⁺ uptake by the islets. Batches of 8-9 islets were preincubated at 37°C for 90 min. Controls were preincubated at 3 mM D-glucose during the whole period and tests were treated with 20 mM D-glucose during the last 5, 10, 30 or 60 min of preincubation. The islets were then transferred to medium supplemented with trace amounts of ⁴⁵Ca²⁺ (7.8 Ci/mol) and incubated for 10 min followed by incubation with LiCl₂ as in Table I. Values are mean values ± S.E. for the number of experiments given in parentheses. ^{*}P < 0.02; ^{**}P < 0.001.

Preincubation with 20 mM D-glucose (min)	Glucose concentration during incubation with ⁴⁵ Ca ²⁺ (mM)	Uptake of labelled calcium (nmol/g dry wt of islets)	Differences from control
Control	3	1.92 ± 0.10 (9)	
5	20	3.01 ± 0.13 (9)	-1.09 ± 0.14
10	20	2.82 ± 0.15 (9)	-0.13 ± 0.16
30	20	2.62 ± 0.14 (9)	-0.39 ± 0.12*
60	20	2.06 ± 0.11 (8)	1.12 ± 0.19**
Control	3	3.18 ± 0.18 (8)	
5	20	2.39 ± 0.10 (9)	0.76 ± 0.12**
10	20	2.37 ± 0.15 (8)	-0.80 ± 0.13**

TABLE III Effects of preincubation with A 23187 on subsequent ionophore-stimulated $^{45}\text{Ca}^{++}$ uptake in islets. Batches of 5 islets were preincubated in basal medium with 3 mM D-glucose at 37°C for 60 min. Controls were preincubated without ionophore and tests were treated with 10 μM A 23187 during the last 30 min or the whole preincubation period. The islets were then transferred to medium containing trace amounts of $^{45}\text{Ca}^{++}$ (7.8 Ci/mol) and incubated for 10 min followed by incubation with LaCl_3 as in Table I. Values are mean values \pm S.E. for the sum of experiments given within parenthesis. $P < 0.02$. The correlation between time of preincubation with A 23187 and $^{45}\text{Ca}^{++}$ uptake was probably significant ($r = 0.553$, $P < 0.05$).

Preincubation time with A 23187 (min)	A 23187 present during incubation with $^{45}\text{Ca}^{++}$	Uptake of labelled calcium (nmol/kg dry wt. of islets)	Differences from control
0	—	1.55 ± 0.15 (6)	-1.50 ± 0.44
0 (control)	+	3.05 ± 0.57 (6)	—
30	+	3.37 ± 0.22 (6)	0.32 ± 0.63
60	+	5.20 ± 0.80 (6)	2.15 ± 1.33

Discussion

The calcium transporting ionophore, A 23187 has previously been shown to decrease glucose oxidation and ATP content in pancreatic islets (Tamarit Rodriguez *et al.* 1971). However the low ionophore concentration employed here (10 μM) does not produce major metabolic changes in the islets (Tamarit Rodriguez *et al.* 1977). The islets used contained more than 90% β -cells (Hellman 1965) and the results are therefore probably representative of this cell type.

Ashby and Speake (1975) reported that A 23187 induced efflux of $^{45}\text{Ca}^{++}$ from prelabelled rat islets, a result confirmed here with the β -cell-rich islets of non-inbred *ob/ob*-mice. Moreover the present results show that A 23187 stimulates the initial uptake of tracer and enhances the equilibrium content of La^{++} nondisplaceable (intracellular) $^{45}\text{Ca}^{++}$. The simplest interpretation of these results is that A 23187 catalyzes fluxes of Ca^{++} across β -cell plasma membranes.

It was previously shown that D-glucose stimulates the uptake of $^{45}\text{Ca}^{++}$ by the La^{++} nondisplaceable calcium pool in *ob/ob*-mouse islets (Hellman *et al.* 1976). However contrast to the results shown here for A 23187 D-glucose did not affect efflux of $^{45}\text{Ca}^{++}$ from prelabelled islets and La^{++} nondisplaceable $^{45}\text{Ca}^{++}$ taken up in response to D-glucose was not significantly reduced during 90 min of incubation in non-radioactive medium (Hellman *et al.* 1976). The present results show that pretreatment of islets with 20 mM D-glucose led to a decrease of the subsequent $^{45}\text{Ca}^{++}$ uptake, suggesting competition between $^{45}\text{Ca}^{++}$ and $^{45}\text{Ca}^{++}$ in an intracellular calcium pool with low mobility. In contrast pretreatment of the islets with 10 μM A 23187 rather increased the subsequent $^{45}\text{Ca}^{++}$ uptake. The clearly different effects of A 23187 and D-glucose on $^{45}\text{Ca}^{++}$ fluxes indicate that the two agents act by different mechanisms in the β -cells and thus add some support to the previous notion (Hellman *et al.* 1976) that D-glucose-stimulated uptake of La^{++} nondisplaceable Ca^{++} may be regulated intracellularly rather than in the islet cell plasma membranes. This idea does not exclude the possibility that D-glucose also regulates Ca^{++} uptake by a La^{++} -displaceable pool located more superficially in the β -cells (Hellman *et al.* 1976).

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Localization of kallikrein and its relation to other trypsin-like esterases in the rat pancreas. A comparison with the submandibular gland

By

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Abstract

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Kallikrein was located by the direct immunofluorescence technique to the granule-containing non-portion of pancreatic acinar cells. For the demonstration of the intracellular distribution of pancreatic kallikrein in the fixation of the gland was necessary. No kallikrein was found in the duct cells or in the islets of Langerhans. Quantitation by a gel radial immunodiffusion showed that the concentration of kallikrein in the pancreas was 132 ± 51 /g/g wet weight, i.e. 1/91 that of the rat submandibular gland. Bz-Arg-OEt-esterases were in the pancreas found as pro-enzyme but as active enzyme in the submandibular gland. Trypsin-like esterases, hydrolyzing α -amino caproic acid naphthyl-1-AS-D HBr (ACA), were found as the active form in both submandibular gland and pancreatic homogenates. The submandibular gland contained per g wet weight 6 times as much ACA-esterase activity as the pancreas. In the submandibular gland, kallikrein and ACA-esterase activity were found together in practically all granular tubular cells. Thus, the granular tubular cell contains kallikrein as well as other trypsin-like enzymes like the ACA-esterase, and in this way comparable to the pancreatic acinar cell. An extraglandular function of kallikrein is suggested for the pancreas in contrast to other kallikrein-containing exocrine organs.

Glandular kallikreins are found in the kidney and the urine, in the pancreas and the pancreatic juice, and in the major salivary glands and the saliva of all mammalian species studied (for review see Nustad *et al* 1978). Kallikreins of exocrine fluids probably are specific for the organ that secretes them (Nustad *et al* 1975). Rat kallikreins of renal and submandibular gland origin have been purified (Nustad and Pierce 1974, Brandtzæg *et al* 1976), and the cellular origin reported (Ørstavik *et al* 1975, Brandtzæg *et al* 1976, Ørstavik *et al* 1977, Ørstavik 1978). In the kidney, kallikrein is present in the distal tubule cells, and in the major salivary glands it is present in the striated duct cells. These two locations have morphological similarities. No kallikrein is found in the acinar cells of the salivary glands, but the granular

ular cells of the rat submandibular gland are particularly rich in kallikrein (Orstavik *et al.* 1977) and in the mouse also other trypsin-like enzymes (Ekfors and Hopsu-Ha *et al.* 1971). The exocrine portion of the pancreas consists mainly of acinar cells, and the duct system is poorly developed with no striated ducts or granular tubules. In the pancreas, trypsin-like enzymes are found in the acinar cells (Hopsu and Glenner 1963), and studies on soluble fractions (Bhoola and Dorsey 1971) indicate that kallikrein is located within the zymogen granules. However the cellular origin of pancreatic kallikrein is unknown. Kallikrein has been purified from the pancreas of several species (for review see Lind *et al.* 1978), and the pancreas is the only organ where kallikrein with certainty has been detected as a pro-enzyme (Fiedler and Werlo 1967).

The purpose of this investigation was to establish the cellular localization of kallikrein (or pro-kallikrein) within the pancreas. Furthermore, its distribution in relation to other trypsin-like enzymes in the pancreas as well as the submandibular gland was studied.

Materials and methods

Animals. Female Sprague-Dawley rats, age 12 to 18 weeks, were used. The animals were killed by blow to head. The submandibular glands and the pancreas were excised and frozen (-20°C) prior to experiments. The animals used in localization studies were anesthetized with nembutal (70 mg per kg m^3) and tracheotomized but undisturbed spontaneous respiration during the experimental period.

Perfusion pressure. The pancreas (as fixed in *see 1*) by perfusion through the superior mesenteric artery using polyethylene cannula (Portex, PP 25) with manually applied pressure and perfusion rate of 1 ml/min, or 2) by isogravimetric perfusion through the abdominal aorta with constant perfusion pressure of 12–18 mm Hg (130 cm high water column), and perfusion rate through the polyethylene cannula (PP 60) of 20 ml/min. When perfusion through the abdominal aorta was performed, pressure was applied to the aorta and vena cava connected to the renal arteries during the perfusion period, and in some animals the renal veins were occluded prior to perfusion. The caval vein was always cut above the kidneys to prevent back-up of back pressure. The gland was first perfused with phosphate buffer saline (PBS, 0.01 M Na-phosphate, pH 7.4, 0.15 M NaCl) containing 5 IU/ml heparin. Buffer perfusion was maintained until the pancreas was pale (after about 2 min), and perfusion with trypsin followed for the next 10 min. The temperature of the perfusates was 22°C . After *in vivo* fixation the pancreas was removed and post-fixed in the same fixative for 20 h (4°C). The submandibular gland was excised during anesthesia, cut into pieces ($3 \times 4 \times 4 \text{ mm}^3$), and fixed *in vitro* for 20 h (4°C). After fixation the tissue was brought through cold water and embedded in paraffin.

Fixative. The fixatives employed were 96% ethanol, 4% formaldehyde in PBS (pH 7.4), 4% buffered formaldehyde saturated with picric acid, or Helly's fluid (a formaline fixative containing dichromate and potassium ferrioxalate). As formalin does not precipitate blood, the wash-out perfusion with buffer was essential for formaldehyde-perfusion.

Immunoelectrophoresis. The immunoglobulin G (IgG) fractions of rabbit antiserum to submandibular gland kallikrein (Brandtzen *et al.* 1976) and sheep antiserum to rat urinary kallikrein (Nustad and Pers 1974) were conjugated with tetramethylrhodamine isothiocyanate (MRTIC) as described by Brandtzen (1974). The conjugates were purified by gel filtration and ion-exchange chromatography. The selected fractions had optical density (OD) ratios ($\text{OD}_{\text{MRTIC}}/\text{OD}_{\text{antiserum}}$) of 2.4 and 2.6 respectively. The working dilution of the conjugates was established by performance testing on tissue sections of the pancreas. The anti-submandibular kallikrein conjugate was employed at an IgG concentration of 0.41 $\mu\text{g}/\text{ml}$, containing 0.12 $\mu\text{g}/\text{ml}$ and 0.7 precipitating units when tested in double diffusion against purified submandibular gland kallikrein. The anti-urinary kallikrein conjugate was used at an IgG concentration of 0.72 $\mu\text{g}/\text{ml}$, containing 0.1 precipitating units when tested in double diffusion against the two kallikreins. Immunological specificity of the staining reaction was controlled by absorption of the conjugates with purified rat urinary kallikrein (Nustad and Pers 1974), 0.20 μg per ml working dilution for the anti-urinary kallikrein conjugate and 0.51 μg per ml of the anti-submandibular kallikrein conjugate or with purified rat submandibular gland

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Abstract

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Fixatives. The fixatives employed were 96% ethanol, 4% formaldehyde in PBS (pH 7.4), 4% buffered formaldehyde saturated with picric acid, or Helly's fluid (a formaline fixative containing dichromate and picric acid, Pierce 1972). As formalin does not precipitate blood, the wash-out perfusion with buffer was normal for formaldehyde-perfusion.

Immunocytochemistry. The haemagglutinin G (IgG) fractions of rabbit antiserum to submandibular gland kallikrein (Brandtzen *et al.* 1976) and sheep antiserum to rat urinary kallikrein (Nustad and Fausa 1974) were conjugated with tetramethylrhodamine isothiocyanate (TRITC) as described by Brandtzen *et al.* (1977). The conjugates were purified by gel filtration and ion-exchange chromatography. The selected fractions had optical density (OD) ratios (OD_{280nm}/OD_{280nm}) of 2.4 and 2.6 respectively. The working dilution of the conjugates was established by performance testing on tissue sections of the pancreas. The anti-submandibular kallikrein conjugate was employed at an IgG concentration of 0.41 µg/ml, containing 0.12 µg/ml and 0.7 precipitating units. It was tested in double diffusion against purified submandibular gland kallikrein (1.05 µg/ml). The sheep anti-urinary kallikrein conjugate was used at an IgG concentration of 0.72 µg/ml, containing 0.1 precipitating units. It was tested in double diffusion against the two kallikreins. Immunological specificity of the staining reaction was controlled by absorption of the conjugates. It was purified rat urinary kallikrein (Nustad and Fausa 1974), 0.20 µg per ml working dilution for the anti-urinary kallikrein conjugate and 0.1 µg per ml of the anti-submandibular kallikrein conjugate or 0.1 µg purified rat submandibular gland

kallikrein (Brandtzaeg *et al.* 1976), 0.21 mg/ml for the anti-urinary and 0.41 mg/ml for the anti-salivary kallikrein conjugate. The anti-salivary kallikrein conjugate was also absorbed with the cross-reacting esterases A and B from the submandibular gland (Brandtzaeg *et al.* 1976), 0.11 mg/ml. Binding specificity was tested by preincubation of the tissue sections with unlabelled antiserum to submandibular gland urinary kallikrein followed by incubation with conjugate diluted in the unlabelled antiserum. The sera were substituted with normal rabbit and sheep sera, respectively for positive controls. On tissue section of the submandibular gland the anti-submandibular gland kallikrein conjugate was used at an IgG concentration of 0.15 mg/ml. The immunological specificity of the fluorescence reaction was controlled by absorption with 0.41 mg purified submandibular gland kallikrein per ml working dilution of the conjugate.

Histochemistry Serial sections (6 µm) adjacent to those stained for kallikrein by immunofluorescence were stained for tryptophan by the Adams DMAB-nitrite method (Pearse 1968) or for trypsin-like esterase activity as described by Hopai and Glenner (1963).

Quantitation of enzymes in gland homogenates. Paired submandibular glands from 7 animals were homogenized separately. Individual pancreas homogenates were produced from 8 animals. These homogenates and kallikrein quantitation by its antigenic activity in a single radial immunodiffusion (SRID) assay or by its Bz-Arg-OEt-esterase activity was performed as described previously (Ørstavik *et al.* 1977). Rabbit antiserum to rat submandibular gland kallikrein was used for the SRID measurements. The antiserum contained 32 precipitating units when tested against a standard submandibular gland homogenate containing 119 mg/ml kallikrein. Trypsin-like esterase activity was measured using the ACA-esterase substrate as described by Hopai and Glenner (1963). The purified submandibular gland enzymes kallikrein esterases A and B (Brandtzaeg *et al.* 1976) as well as purified urinary kallikrein (Nustad and Pierce 1976) were tested for ACA-esterase activity. Bovine trypsin was used as standard, and ACA-esterase activity was expressed in trypsin units. One trypsin unit (TU) was defined as the amount of free naphthol-AS released per min by 1 µg of the trypsin standard, measured by the optical density at OD_{540nm}.

Pancreatic pro-enzymes were activated either by auto-digestion (24 h, 22°C) or by trypsin-activation (equal volume of trypsin (1 mg/ml in 0.05 M TRIS/HCl buffer pH 7.75 30 min, 22°C) was added to the pancreas homogenate). Trypsin activation was stopped with soybean trypsin inhibitor (SBTI) (10 mg, final concentration). The activated homogenate was then left for 30 min (22°C) before testing for esterase activity. Trypsin activation was also done on the submandibular gland homogenates.

Determination of total protein. Total protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Drugs: Heparin (Leo, Ballerup, Denmark), Fast Garnet GBC® (Sigma), Trypsin 1 250® (Difco).

Results

Immunohistochemical localization of kallikrein In the pancreas, kallikrein was localized within the acinar cells (Fig. 1). The kallikrein-specific fluorescence (Fig. 1c) showed a similar distribution as the DMAB-positive secretory granules (Fig. 1e) and was found in all acinar cells. No kallikrein was observed in the cytoplasm of the ductal cells, whereas some kallikrein-containing material was seen in the duct lumen and adhering to the luminal wall. The islets of Langerhans were devoid of kallikrein. This location of kallikrein was obtained by use of both the anti-salivary and the anti-urinary kallikrein conjugates. However the latter was a weaker antiserum and produced faint fluorescence. Staining was completely blocked by absorbing the conjugates with purified submandibular gland urinary kallikrein (Fig. 1a and b). Absorption with submandibular gland esterases A and B did not alter the fluorescence reaction. The fluorescence reaction was abolished by preincubation of the tissue sections with the antiserum to kallikrein but not with normal sera.

For the immunofluorescence studies on the pancreas, ethanol was the only fixative which gave a strong fluorescence against a dark background. With the other fixatives employed (formaldehyde, formaldehyde with picric acid, and Helly's fluid) there was little difference between specific and non-specific staining and no brilliant fluorescence was seen in the

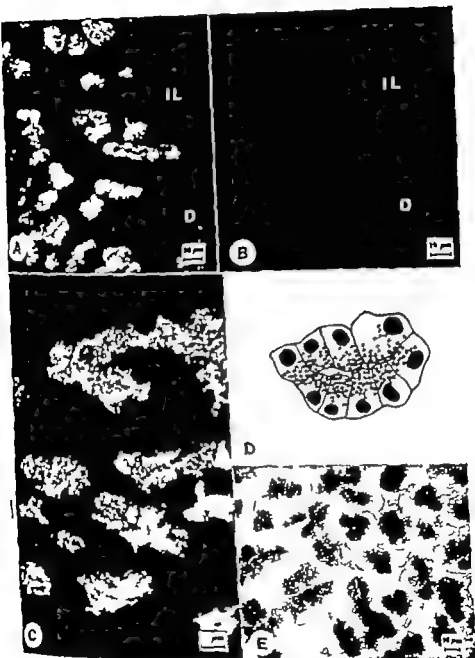


Fig. 1. Immunohistochemical localization of kallikrein in the rat pancreas fixed *in situ* with ethanol. Kallikrein reacts in the lateral part of all acinar cells (A and C) as visualized by stars in the line-drawing of pancreatic islets (D). Kallikrein shows granular distribution as DMAR-positive granular material in formal fixed tissue (E). The specificity of the fluorescence reaction is demonstrated in the microphotographs of identical areas of two adjacent sections: one reacted with unabsorbed antiserum; duodenal gland kallikrein conjugate (A) and the other with conjugate absorbed with purified porcine urinary kallikrein (B). The exposure times for Fig. A and B are 15 sec. IL—cells of Langerhans; D—duct.

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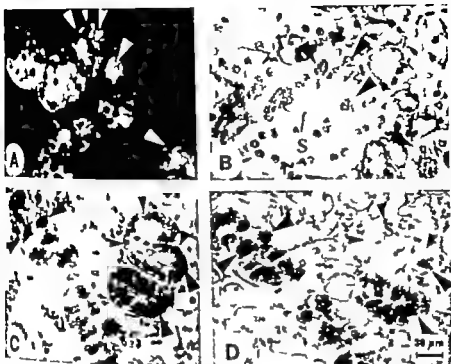


Fig. 2. Distribution of ACA-esterase (B and D) of the rat submandibular gland in relation to kallikrein (A) and trypsinogen (C). Small arrows point to non-granular duct cells whereas large arrows point at granular tubular cells. Note the presence of kallikrein and ACA-esterase in the same granular tubular cells (A and B), and the similar distribution of esterase and trypsinogen (C and D). S—striated duct.

Total pancreatic and submandibular gland ACA-esterase activity per g wet weight is given in Table I. Pancreatic ACA-esterase activity was not significantly altered by gland homogenate activation (Table I). Similar to the submandibular gland Bz-Arg-OEt-esterase activity (Ørstavik and Gustvik 1977) the difference between the ACA-activity of paired glands was small compared to that between different animals, *i.e.* $5.5 \pm 7.1\%$ versus $33.3 \pm 21.5\%$. The contribution of various submandibular gland esterases to total gland Bz-Arg-OEt-esterase activity and their specific Bz-Arg-OEt-esterase activities are known (Brandtzaeg *et al.* 1976), as well as their specific ACA-activity (Table II). It was thus possible to give an estimation of the percentage of total submandibular gland ACA-activity contributed by other esterases than kallikrein (Table II).

Discussion

The direct immunofluorescence technique has previously been used to localize kallikrein in other exocrine organs (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976, Ørstavik *et al.* 1976, Ørstavik 1977). The reliability of these results was substantiated by the corresponding change in kallikrein fluorescence and kallikrein enzyme activity in the developing rat submandibular

luminal part of the acinar cells. The *in situ* fixation was absolutely necessary to demonstrate the intracellular distribution of pancreatic kallikrein. Also, fixation had to be achieved rapidly. When fixation was not satisfactory the fluorescence observed was weaker and more uniformly spread out over the whole acinar cell. A satisfactory fixation throughout the entire gland depended on the wash-out effect of the buffer perfusion to prevent precipitation of blood by all fixatives except formaldehyde. When a retrograde perfusion through the abdominal aorta was used, occlusion of the renal vessels improved fixation.

In the submandibular gland kallikrein showed the same localization as previously (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976), *i.e.* in the granular tubular and striated cells. Compared to the other fixatives employed, 4% formaldehyde saturated with picric acid resulted in a brilliant fluorescence with only little nonspecific back-ground staining and individual kallikrein-containing granular tubular cells could easily be differentiated. All cells with a typical granular appearance contained kallikrein, even though the intensity varied from cell to cell.

Localization of ACA-esterase and its relation to kallikrein. ACA-esterase activity was demonstrated in the granular tubular cells of the submandibular gland. No reaction was detected in the striated duct cells whereas some traces could occasionally be seen within the duct lumen. No esterase activity was detected in the acini. Positive esterase activity was only observed in formaldehyde-fixed tissue, rendering comparison with kallikrein-positive cells difficult since in these sections cells were not readily identified. However when examining distal portions of the granular tubules with only few granular tubular cells, it could be seen in neighboring sections that both kallikrein and ACA-esterase as well as tryptophan-rich substance were present within the same cell (Fig. 2). Moreover, practically all cells of the granular type were found to contain tryptophan, ACA-esterase, as well as kallikrein using the most suitable fixative for the three, *i.e.* Helly's fluid, formaldehyde, and formaldehyde containing picric acid respectively. By prolonging the incubation period for ACA-esterase detection, increased intensity in positive cells was observed, but the number of positive cells remained the same.

In pancreatic tissue sections no ACA-esterase activity was observed. The whole section was stained light brown but there was no sign of red precipitate within the cells. Also, examining the sections in a Leitz Ortholux microscope with a Ploem-type vertical illuminator and interference filters for narrow-band excitation of red fluorescence, which in the submandibular gland improved detection of esterase activity, did not reveal any positive reaction in the pancreas.

Quantitation of esterases in the submandibular gland and pancreas. The amount of kallikrein in the submandibular gland and pancreas was quantitated by SRID and Bz Arg-OEt-esterase activity. The results are given in Table I. In the pancreas, Bz Arg-OEt-esterases were found as pro-enzymes. Activation of pancreas pro-enzymes yielded somewhat less esterase activity than expected from calculations of kallikrein esterase activity based on SRID quantitation and the specific pancreatic kallikrein Bz Arg-OEt-esterase activity (345 EU/mg, Højima *et al.* 1977b). However, in the submandibular gland trypsin and SBTI showed some inhibitory action, and such inhibitory effect may explain the low values in the pancreas.

TABLE II The specific Nz-Arg-OMe - and ACA-esterase activity of some purified submandibular gland extracts, and the part of the total gland esterase activity which these enzymes represent.

	Nz-Arg-OMe-esterase activity			ACA-esterase activity		
	EU/mg prot	% of total gland act.	EU/gland	TU/mg prot	Calculated TU/gland	Calculated % of total gland act.
Rat submandibular gland esterase						
Esterase A ₁	95 ^a	7.3	153	13	21	1.2
Esterase A ₂	123 ^a	3.6	76	114	41	2.3
Esterase B	304 ^a	22.3	469	141	217	12.3
Kallikrein	599 ^a	66.7	1401	122	285	16.2
Other esterases		0				68.0
Total gland act.	7 ^b	100	2101		1762	100
			± 682		± 712	
Rat pancreatic kallikrein						
Trypsin (Difco)	815			130		
	5			1000		

From Brandstetter *et al.* 1976

From Gostovik and Gostovik 1977

See also Table I where similar calculations were made for each gland separately

In the pancreas kallikrein exists as a pro-enzyme (Fiedler *et al.* 1970, Hojima *et al.* 1974). Pro-kallikrein and kallikrein (partially purified) show immunological identity and are not detectable with the antiserum employed in the fluorescence study (Proud *et al.* 1977). As kallikrein antigen, but not kallikrein esterase activity could be detected in non-activated tissue homogenates, it is possible that kallikrein detected immunohistochemically actually represents pro-kallikrein. However the effect of tissue fixation and processing for immunohistochemistry on enzyme configuration is not known. The localization of pro-kallikrein can only be ascertained by means of an antiserum specific for pro-kallikrein determinants.

The ACA-esterase reaction observed in the rat submandibular gland and the pancreas was shown not to be specific for one particular enzyme. Kallikrein and esterases A and B all have the ability to hydrolyze the ACA-ester although to a varying degree. However in the submandibular gland 68% and in the pancreas 98% of the total ACA-activity represent unidentified enzymes. Thus, the bulk of ACA-activity may very well represent a trypsin-like enzyme different from the esterases tested. Since in the pancreas, ACA-activity was not significantly influenced by pro-enzyme activation, the (se) unidentified ACA-esterase(s) are probably not trypsin which in the pancreas exists as a pro-enzyme.

Kallikrein and ACA-esterase are in the submandibular gland located within the same granular tubular cells as revealed by serial tissue sections. Moreover the presence of the two enzymes in most granular tubular cells excludes the possibility that this is due to kallikrein ACA-activity as kallikrein makes up only 19% of the total gland ACA-activity. In the pancreas, kallikrein shows a distribution similar to the zymogen granules as visualized by the DMAB-staining. Kallikrein(ogen) and trypsinogen are found in isolated zymogen granules (Bhoola and Dorey 1971). ACA-esterase activity has also been found in the rat

TABLE I Kallikrein (antigen), Bz Arg-OEt and ACA-esterase activity in the submandibular gland or pancreas. Gland homogenates were treated by trypsin-activation, soybean trypsin inhibitor (SBTI), or autoactivation (see Method). Pro-enzymes were not detected in the submandibular gland whereas in the pancreas Bz Arg-OEt-esterases were found as pro-enzymes. Pancreatic ACA-esterase activity was not altered by gland homogenate activation. () number of glands.

	Submandibular gland				Pancreas			
	Non-activated	Trypsin + SBTI	SBTI	Calculated act. representing kallikrein ^a	Non-activated	Auto-activated	Trypsin + SBTI	Calculated act. representing kallikrein ^a
Kallikrein (SRID) $\mu\text{g/g ww}$	11 980 $\pm 2 253$ (14)	n.d.	n.d.		13 ± 51 (6)	n.d.	n.d.	
Bz-Arg-OEt est. act. EU/g ww	11 859 ± 619 (5)	2 697 ± 873 (5)	4 871 ± 6.4 (5)	7 012 ± 962 (5)	0 (6)	40.6 ± 6.5 (5)	16.1 ± 3.9 (6)	44.9 ± 20.5 (6)
ACA est. act. TU/g ww	8 120 $\pm 3 422$ (14)	n.d.	n.d.	1 432 ± 190 (14)	1 363 ± 778 (5)	1 003 ± 650 (6)	1 820 ± 979 (4)	164 ± 7.5 (6)
				[19.6 ± 6.0] ^b [of total act.]				[1.8 ± 0.4] [of total act.]

Gland kallikrein concentrations were obtained by SRID (see methods). Specific Bz-Arg-OEt-esterase activity of submandibular gland kallikrein (599 EU/mg) and pancreatic kallikrein (345 EU/mg) has been given by Brandtzaeg *et al.* (1976) and by Hoffma *et al.* (1977b) respectively. Specific ACA-esterase activity of submandibular gland kallikrein is given in Table II. For pancreatic kallikrein the mean of submandibular gland and urinary kallikrein specific ACA-activity (122 and 130 TU/mg protein) was used as it was assumed that pancreatic kallikrein did not differ much from these two.

^a The kallikrein percentage of total ACA-esterase activity could also be calculated by kallikrein Bz-Arg-OEt-quantifications as 67% of total Bz-Arg-OEt-esterase activity is due to kallikrein (Brandtzaeg *et al.* 1976). This gave a kallikrein percentage of total ACA-activity of 18.4 ± 2.4 which is equal to that calculated from the SRID-kallikrein concentrations.

gland (Ørstavik and Gautvik 1977, Ørstavik *et al.* 1977). Also in the cat submandibular gland immunofluorescence studies demonstrated kallikrein in the duct system (Hoffma *et al.* 1977a). The localization obtained in the present study was controlled by absorbing the fluorescent conjugate with purified kallikrein from urine and the submandibular gland. The identity of these two antigens was established by their kinin-generating ability then oxytocic as well as hypotensive activity and the esterase activity compared to the caseinolytic activity (Nustad and Pierce 1974, Brandtzaeg *et al.* 1976). Moreover antisera produced against these two different kallikreins both gave the same cellular localization of pancreatic kallikrein, *i.e.* in the acinar cells. Despite the similarity in amino acid composition between pancreatic kallikrein and trypsin (Fiedler 1976), these two enzymes (partially purified) did not show immunological cross-reactivity when reacted in double diffusion or in a radioimmunoassay with the same antiserum against rat submandibular gland kallikrein as used in the present study (Proud *et al.* 1977). The presence of trypsin in the acinar cells should therefore not disturb immunohistochemical detection of pancreatic kallikrein. Moreover when the antiserum was reacted against crude pancreas homogenate, only one precipitin line could be detected, and this antigen was immunologically identical to the partially purified kallikrein (Proud *et al.* 1977). Furthermore, absorption of the conjugate with cross-reacting antigens from the submandibular gland did not influence the fluorescence reaction.

Table II The specific Br-Arg-OMe and ACA-esterase activity of some purified submandibular gland enzymes, and the part of the total gland esterase activity which these enzymes represent.

	Br-Arg-OMe-esterase activity			ACA-esterase activity		
	EU mg prot	of total gland act.	EU gland	TU mg prot	Calculated TU gland	Calculated of total gland act.
Submandibular gland esterase:						
Enzyme A ₁	99 ^a	7.3	153	13	1	1.2
Enzyme A ₂	1.3 ^a	3.6	76	114	41	3
Enzyme B	304 ^a	22.3	469	141	17	12.3
Kallikrein	599 ^b	66.7	1 401	122	793	16
Other enzymes		0				68.0
Total gland act.	7 ^a	100	2 101		1 76	100
			64%		71	
In serum kallikrein	115			130		
From (Dole)	5			1 000		

^a From Brändberg *et al.* 1976

^b From Petersén and Gærnér 1977

In the Table I have similar calculations are made for each gland separately

In the pancreas kallikrein exists as a pro-enzyme (Fiedler *et al.* 1970, Hojima *et al.* 1971). Pro-kallikrein and kallikrein (partially purified) show immunological identity and are not distinguishable with the antiserum employed in the fluorescence study (Proud *et al.* 1971). As kallikrein antigen, but not kallikrein esterase activity could be detected in non-acid tissue homogenates, it is possible that kallikrein detected immunohistochemically actually represents pro-kallikrein. However the effect of tissue fixation and processing for immunohistochemistry on enzyme configuration is not known. The localization of pro-kallikrein can only be ascertained by means of an antiserum specific for pro-kallikrein components.

The ACA-esterase reaction observed in the rat submandibular gland and the pancreas was shown not to be specific for one particular enzyme. Kallikrein and esterases A and B all have the ability to hydrolyze the ACA-ester although to a varying degree. However in the submandibular gland 68% and in the pancreas 98% of the total ACA-activity represent unidentified enzymes. Thus, the bulk of ACA-activity may very well represent a trypsin-like enzyme different from the esterases tested. Since in the pancreas, ACA-activity was not significantly influenced by pro-enzyme activation, the (se) unidentified ACA-esterase(s) are probably not trypsin which in the pancreas exists as a pro-enzyme.

Kallikrein and ACA-esterase are in the submandibular gland located within the same granular tubular cells as revealed by serial tissue sections. Moreover the presence of the same enzymes in most granular tubular cells excludes the possibility that this is due to kallikrein ACA-activity as kallikrein makes up only 19% of the total gland ACA-activity. In the pancreas, kallikrein shows a distribution similar to the zymogen granules as visualized by the DMSA-staining. Kallikrein(ogen) and trypsinogen are found in isolated zymogen granules (Ribolet and Dorey 1971). ACA-esterase activity has also been found in the rat

TABLE I. Kallikrein (antigen), Bz Arg-OEt and ACA-esterase activity in the submandibular gland and pancreas. Gland homogenates were treated by trypsin-activation, soybean trypsin inhibitor (SBTI), or autoactivation (see Methods). Pro-enzymes were not detected in the submandibular gland whereas in the pancreas Bz Arg-OEt-esterases were found as pro-enzymes. Pancreatic ACA-esterase activity was not altered by gland homogenate activation. () number of glands.

	Submandibular gland				Pancreas			
	Non-acti- vated	Trypsin + SBTI	SBTI	Calculated act. representing kallikrein ^a	Non-acti- vated	Auto-acti- vated	Trypsin + SBTI	Calculated act. representing kallikrein ^a
Kallikrein (SRID) μg/g w w	11 980 ± 2 253 (14)	n.d.	n.d.		132 ± 51 (6)	n.d.	n.d.	
Bz Arg-OEt est. act. EU/g w w	11 859 ± 2 619 (5)	2 697 ± 875 (5)	4 872 ± 624 (5)	7 012 ± 962 (5)	0 (6)	40.6 ± 6.5 (5)	16.1 ± 2.9 (6)	44.9 ± 20.5 (6)
ACA est. act. TU/g w w	8 120 ± 3 422 (14)	n.d.	n.d.	1 432 ± 190 (14)	1 363 ± 778 (5)	1 003 ± 650 (6)	1 820 ± 979 (4)	16.4 ± 7.5 (6)
				[19.6 ± 6.0 of total act.] ^b				[1.3 ± 0.4 of total act.]

TABLE II The specific Br-Ary-OEt- and ACA-esterase activity of some purified submandibular gland enzymes, and the part of the total gland esterase activity. Each these enzymes represent

	Br-Ary-OEt-esterase activity			ACA-esterase activity		
	EU mg prot	of total gland act.	EU gland	TU mg prot	Calculated TU gland	Calculated of total gland act.
Submandibular gland enzymes						
Esterase A ₁	95 ^a	7.3	153	13	1	1.2
Esterase A ₂	1.5 ^a	3.6	76	114	41	3.3
Esterase B	304 ^a	22.3	449	141	17	1.3
Kallikrein	999 ^a	66.7	1401	122	283	16.0
Other enzymes		0				68.0
Total gland act.	7 ^b	100	2101		176.0	100
			± 602		712	
Br-enzyme Kallikrein	819			130		
Trypsin (Difco)	5			1000		

^a From Brandeney *et al.* 1976

^b From Gosselin and Gosselin 1977

In the Table I, here similar calculations were made for each gland separately

In the pancreas kallikrein exists as a pro-enzyme (Fiedler *et al.* 1970, Hojima *et al.* 1971). Pro-kallikrein and kallikrein (partially purified) show immunological identity and are not distinguishable with the antiserum employed in the fluorescence study (Proud *et al.* 1971). As kallikrein antigen, but not kallikrein esterase activity could be detected in non-activated tissue homogenates, it is possible that kallikrein detected immunohistochemically really represents pro-kallikrein. However the effect of tissue fixation and processing for immunohistochemistry on enzyme configuration is not known. The localization of pro-kallikrein can only be ascertained by means of an antiserum specific for pro-kallikrein fractions.

The ACA-esterase reaction observed in the rat submandibular gland and the pancreas was shown not to be specific for one particular enzyme. Kallikrein and esterases A and B all have the ability to hydrolyze the ACA-ester although to a varying degree. However in the submandibular gland 68% and in the pancreas 98% of the total ACA-activity represent unidentified enzymes. Thus, the bulk of ACA-activity may very well represent a trypsin-like enzyme different from the esterases tested. Since in the pancreas, ACA-activity was not significantly influenced by pro-enzyme activation, the (se) unidentified ACA-esterase(s) are probably not trypsin which in the pancreas exists as a pro-enzyme.

Kallikrein and ACA-esterase are in the submandibular gland located within the same granular tubular cells as revealed by serial tissue sections. Moreover the presence of the two enzymes in most granular tubular cells excludes the possibility that this is due to kallikrein ACA-activity as kallikrein makes up only 19% of the total gland ACA-activity. In the pancreas, kallikrein shows a distribution similar to the zymogen granules as visualized by the DNAB-staining. Kallikrein(ogen) and trypsinogen are found in isolated zymogen granules (Booth and Dorey 1971). ACA-esterase activity has also been found in the rat

pancreatic zymogen granules (Lagunoff *et al* 1962) and in the acinar cells of the human pancreas (Hopsu and Glenner 1963). However we did not succeed in demonstrating ACA activity in the rat pancreatic tissue sections. Since in the rat, the pancreas contains on about 1/6 per g wet weight of the submandibular gland ACA-activity the negative result may be due to a concentration below detection limit in the pancreas. The *in vivo* formaldehyde fixation of the pancreas was rapid and complete, therefore autolytic destruction of zymogen granules probably was not the reason for the negative results, even though rapid fixation was critical for kallikrein detection. However in spite of the failure to demonstrate the cellular distribution of ACA-esterase activity in the present study it seems reasonable to assume that kallikrein as well as other trypsin like enzymes, like the ACA-esterase, located in the acinar cells of the pancreas as they are in the granular tubular cells in the rat submandibular gland.

In the major salivary glands, kallikrein is found within the duct system, and in the kidney in the distal tubules which resemble the striated duct of the salivary glands. In the exorbital lacrimal gland no kallikrein was detected (Ørstavik 1978) and this gland like the pancreas is devoid of striated duct-like structures. Thus the location of kallikrein in the pancreas is different from that seen in other exocrine organs investigated. However the acinar cells of the pancreas resemble the granular tubular cells of the rat submandibular gland both have ample amounts of secretory granules containing enzymes like kallikrein and other trypsin like esterases which upon cellular degranulation are secreted into the duct lumen. The physiological importance and the site of action of kallikrein remain unresolved questions. However the presence of kallikrein in the pancreas solely as a pro-enzyme is characteristic for this organ. Pro-kallikrein probably is activated by trypsin which itself is activated mainly in the duodenum. Therefore, it seems as if structures in the gut may be the location for pancreatic kallikrein function. Functional analyses of kallikrein may thus be facilitated by comparisons of kallikrein action in the duodenum, the striated ducts of the salivary glands, and the distal tubules of the kidney.

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Attraction of nerve fiber outgrowth from sympathetic ganglia to heart auricles in tissue culture

By

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Abstract

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Sympathetic chain ganglia of 3-day-old rats were cultured in Rose chambers for 5 days. Each chamber contained either two ganglia or a ganglion and a piece of heart atrium. Visual examination failed to show an increased density or length of nerve fibers growing towards the other explant. However, actual measurements showed that the length of nerve fiber outgrowth towards the atrium explant was about 10% longer than the mean outgrowth to all directions, the difference being statistically significant ($P < 0.0001$). In ganglion-ganglion pair cultures the nerve fiber outgrowth towards the other ganglion tended to be shorter than that to other directions. It is tentatively concluded that atrium explants exert a growth-promoting influence on sympathetic nerve fibers in cultures, possibly due to diffusion of growth-promoting substances from the atrium explant.

In a thorough discussion of growth and maturation of neurons, Jacobson (1970) points out that direct evidence of chemotactic guidance of nerve fiber growth is not yet available. However, it has recently been reported by Chamley *et al.* (1973) that more nerve fibers from explants of rat sympathetic ganglia *in vitro* grow preferentially to nearby explants of normally densely innervated autonomic effectors, such as the heart and vas deferens, as compared with fiber growth to explants of normally sparsely innervated organs, such as the kidney, uterus, ureter or lung. In a subsequent study Chamley and Dowel (1975) reported, moreover, that sympathetic nerves can distinguish between different parts of the same organ with different densities of innervation *in vitro*: nerve fibers from explants of cultured sympathetic ganglia grew mainly to atrium explants, although ventricle explants were present at the same distance.

Chamley *et al.* (1973) and Chamley and Dowel (1973) based their observations on visual assessment of nerve fiber growth without any measurements. Since our own visual observations on joint cultures of sympathetic ganglia and target organs failed to show any clear preferential growth towards densely innervated organs, we decided to carry out a quantitative study in which visual examination was supplemented by quantitative measurements of

length of nerve fiber growth in different directions from the ganglion explant. Such measurements show without ambiguity how far fibers grow from the ganglion explant towards the target, as compared with other directions. Moreover they make possible the use of statistical methods to assess the significance of the directional growth, if this seems to be proper.

Materials and methods

Culture method

Nale myelinated thoracic and lumbar human ganglia and small pieces of heart atrium of about 3-day-old Sprague Dawley rats were cultured in Rowe (1954) chambers. The culture method has been described in previous papers (Zifchock *et al.* 1972 a, b). It is the same as that used by Chanley *et al.* (1973) and Chanley and David (1974), except for the mode of arrangement of the explants on the coverslip to be described later.

After aseptic removal under binocular microscope the myelinated chain dissected in 11 \times 1 balanced aluminum (Hanks and Wallace 1949) with 10% fetal calf serum and 100 000 i.u. penicillin sodium & 64000 i.u. streptomycin. The ganglia were treated with 0.125 trypsin for 30 min at 37°C, and rinsed in the balanced salt solution. The connective explants and the ganglia were then placed on coverslips previously coated with collagen (Eliasson and Gey 1956, Hanzelka and Koenigsberg 1966) and covered with strip of 10 \times 10 mm polyethylene (Rowe *et al.* 1954). The chambers were supplied with Medium 199 (Salk *et al.* 1954) explants with 20% (v/v) fetal calf serum, 50 i.u. insulin, 100 000 i.u. penicillin sodium & 64 000 i.u. streptomycin. No nerve growth factor was used. The chambers were kept at 37°C in incubator and in pH of the medium was set at 7.2 by constant flow of 5% carbon dioxide air bubbled through water.

Arrangement of explants in chambers

F.J. Chanley *et al.* (1973) cultured 2 rows of different organs on both sides of row of ganglia, about only 2 explants in each chamber to ascertain the presence or absence of preferential growth towards the other explant. In each chamber ganglion was cultured together with another ganglion or piece of heart atrium, about the same size as the ganglion and taken from the same animal. Atrium was used because this organ as reported by Chanley *et al.* (1973) to readily attract nerve fiber growth. The distance between the explants (from margin to margin) is about 1.5-2 mm.

After setting up the culture chambers, these were left for 3 day undisturbed in the incubator so as to allow establishment of possible chemotactic gradients forming between the explants. During this time the media was horizontal, the explants above it.

Measurement of nerve fiber growth

After 3 days the cultures were examined under phase contrast microscope and the extent of fiber growth from the explant was measured in 4 perpendicular directions by moving the specimen in the end of the microscope stage and reading the Vernier scales. These measurements were carried out at 125 magnification (10, N.A. 0.40) by which the fine nerve fibers were well visible. The outlines of the explants and the outgrowth around them were also drawn with the aid of drawing tube and dark field illumination.

Results

General observations

A rich network of nerve fibers always grew out of the ganglion explant, as is shown at low magnification in Fig. 1. Under the conditions of the present study nerve fibers and spindle shaped glial cells grew together. Thus, neither naked nerve fibers without glial cells nor glial cells without nerve fibers were seen outside the outgrowth border which they together formed, with rare exceptions. This is illustrated in Fig. 2, which shows typical phase contrast photographs from the periphery of the ganglion outgrowth. Nerve fibers and glial cells were easily recognized at the magnification employed for the measurements.

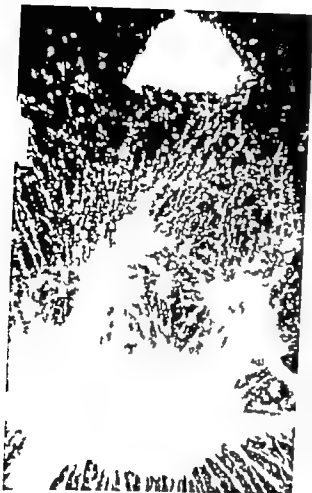


Fig. 1 Low power dark field photomicrograph of a "fused" 5-day ganglion and atrium joint culture. A dense network of nerve fibers and supporting cells grows out of the ganglion explant in the lower part of the figure, this outgrowth extends beyond the atrium explant in the upper part of the figure. Groups of migrating nerve cells have become detached from the explant and form pseudopodia and an island above it. Magnification $\times 20$.

As a rule, no outgrowth occurred from the atrium explants. In the rare occasions when this happened there was no difficulty in discriminating this outgrowth from the nerve fiber outgrowth of the ganglion. Therefore, it was possible to measure the length of nerve fiber outgrowth towards the atrium explant even when the nerve fibers actually grew up to the atrium explant and past it.

If the nerve fibers from the ganglia in pair cultures were long enough so that they met and intermingled between the explants, it was impossible to measure the distance of outgrowth to that direction.

Nerve fiber outgrowth to different directions

In Fig. 3 are superimposed several drawings made of 5 day cultures, showing the ganglion explant in the center, the atrium explant in the periphery and the outlines of the fiber outgrowth around the ganglion. A circle is drawn around the center of the ganglion explant, the mean distance to the center of the atrium explant forming a radius. It is obvious from the drawing that the distance of outgrowth around the ganglion greatly varied from one explant to another. The outgrowth towards the atrium explant, marked 0° is approximately similar to that in any other direction, of which 90° , 180° and 270° have been indicated because

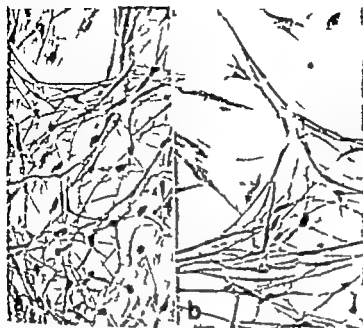


Fig. 3. Two contact photomicrographs of peripheral areas of outgrowth from a ganglion explant cultured in 3 days. a) Fine nerve fibers accompanied by spindle shaped glial cells form a typical dense network. b) Peripheral border of the nerve fiber outgrowth. A growth cone can be seen on the upper left part of the figure. The nerve fibers are closely associated with glial cells. Flat supporting cells, unlike those of nerve fibers and glial cells, can easily be differentiated from these. Objective 10, N.A. 0.40. Reproduced 100.

nerve outgrowth measurements were carried out in these directions. It can also be seen from Fig. 3 that the nerve fiber outgrowth in many cases reached the atrium explant.

Visual examination of the cultures failed to show that the nerve fibers growing towards the other explant, atrium or another ganglion, had been longer or more numerous than those growing to other directions.

Quantitative nerve fiber outgrowth measurements

Table I shows the distance of nerve fiber outgrowth in mm to the 4 perpendicular directions indicated in Fig. 3. The distance of outgrowth was measured both from the center of the explant and from the border of the explant. The whole material is shown in the upper third of Table I, in the center third are the "fused" cultures, whose outgrowth contacted the other explant or the outgrowth from it, and in the lower third are the "separate" cultures, whose outgrowth did not reach the other explant or its outgrowth.

Because of intermingling of nerve fibers between paired ganglion cultures, it was not possible to measure the extent of growth at direction of 0° in all cultures, and the mean for all cultures is omitted, since it is not representative of the whole material. The upper data still serve to show that the extent of nerve fiber outgrowth to directions 90°-180° or 180°-90° in ganglion and atrium pair cultures was closely similar to that in paired ganglion cultures. The mean distance of outgrowth from the center of explant towards the atrium

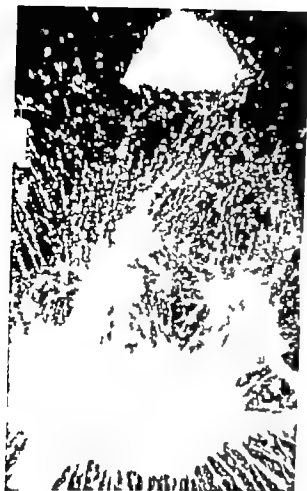


Fig. 1 Low power dark field photomicrograph of "fused" 5-day ganglion and atrium culture. A dense network of nerve fibers and supporting cells grows out of the ganglion explant in the lower part of the figure, as this outgrowth extends beyond the atrium explant in the upper part of the figure. Groups of migrating nerve cells have been detached from the explant and form a peninsula and an island above it. Magnification 20.

As a rule, no outgrowth occurred from the atrium explants. In the rare occasions when this happened there was no difficulty in discriminating this outgrowth from the nerve fiber outgrowth of the ganglion. Therefore, it was possible to measure the length of nerve fiber outgrowth towards the atrium explant even when the nerve fibers actually grew up to the atrium explant and past it.

If the nerve fibers from the ganglia in pair cultures were long enough so that they met and intermingled between the explants, it was impossible to measure the distance of outgrowth to that direction.

Nerve fiber outgrowth to different directions

In Fig. 3 are superimposed several drawings made of 5 day cultures, showing the ganglion explant in the center, the atrium explant in the periphery and the outlines of the fiber outgrowth around the ganglion. A circle is drawn around the center of the ganglion explant, the mean distance to the center of the atrium explant forming a radius. It is obvious from the drawing that the distance of outgrowth around the ganglion greatly varied from one explant to another. The outgrowth towards the atrium explant, marked 0° is approximately similar to that in any other direction, of which 90°, 180° and 270° have been indicated because

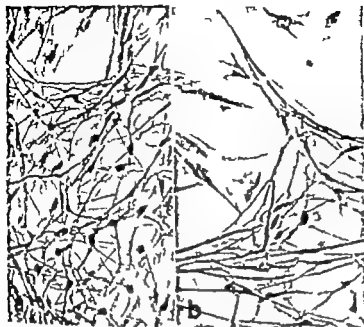


Fig. 2. Phase contrast photomicrographs of peripheral areas of outgrowth from ganglion explant cultured in 2 hrs. a) Fine nerve fibers interwoven by spindle shaped glial cells form typical dense network. b) Terminal border of the nerve fiber outgrowth. A growth cone can be seen at the upper left part of the explant. The nerve fibers are closely associated with glial cells. Flat supporting cells, visible above the network of nerve fibers and glial cells, can easily be differentiated from these. Objective 10, N.A. 0.40. Magnification 160.

and outgrowth measurements were carried out in these directions. It can also be seen from Fig. 3 that the nerve fiber outgrowth in many cases reached the atrium explant.

Visual examination of the cultures failed to show that the nerve fibers growing towards the other explant, atrium or another ganglion, had been longer or more numerous than those growing to other directions.

Nerve fiber outgrowth measurements

Table I shows the distance of nerve fiber outgrowth in mm to the 4 perpendicular directions measured in Fig. 3. The distance of outgrowth was measured both from the center of the neuron explant and from the border of the explant. The whole material is shown in the first third of Table I, in the center third are the "fused" cultures, whose outgrowth contacted the other explant or the outgrowth from it, and in the lower third are the "separate" cultures, whose outgrowth did not reach the other explant or its outgrowth.

Because of intermingling of nerve fibers between paired ganglion cultures, it was not possible to measure the extent of growth at direction of 0° in all cultures, and the mean for all cultures is indicated, since it is not representative of the whole material. The upper part will serve to show that the extent of nerve fiber outgrowth to directions 90°-180° or 270° in ganglion and atrium pair cultures was closely similar to that in paired ganglion cultures. The mean distance of outgrowth from the center of explant towards the atrium

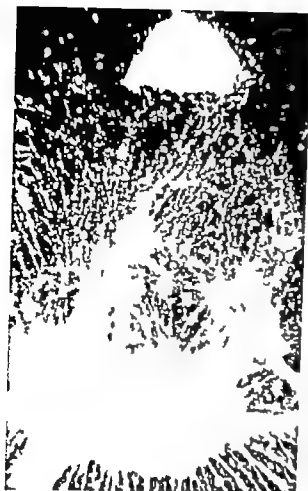


Fig. 1. Low power dark field photomicrograph of a "fused" 5-day ganglion and atrium explant culture. A dense network of nerve fibers and supporting cells grows out of the ganglion explant (the lower part of the figure); this outgrowth extends beyond the atrium explant in the upper part of the figure. Groups of migrating nerve cells have been detached from the explant and form a peninsula and an island above it. Magnification $\times 20$.

As a rule, no outgrowth occurred from the atrium explants. In the rare occasions when this happened there was no difficulty in discriminating this outgrowth from the nerve fiber outgrowth of the ganglion. Therefore, it was possible to measure the length of nerve fiber outgrowth towards the atrium explant even when the nerve fibers actually grew up to, and past it.

If the nerve fibers from the ganglia in pair cultures were long enough so that they met and intermingled between the explants, it was impossible to measure the distance of outgrowth in that direction.

Nerve fiber outgrowth to different directions

In Fig. 3 are superimposed several drawings made of 5 day cultures, showing the ganglion explant in the center, the atrium explant in the periphery, and the outlines of the fiber outgrowth around the ganglion. A circle is drawn around the center of the ganglion explant, the mean distance to the center of the atrium explant forming a radius. It is obvious from the drawing that the distance of outgrowth around the ganglion greatly varied from one explant to another. The outgrowth towards the atrium explant, marked 0° , is approximately similar to that in any other direction, of which 90° , 180° and 270° have been indicated because

TABLE I. Nerve fiber outgrowth (mm) from sympathetic ganglion explant towards another explant (0°) and towards other directions in 5 day pair cultures.

Tissue type	Number of cultured ganglia	Direction of growth measurement (°)	Distance of outgrowth from center of explant (mm)		Distance of outgrowth from border of explant (mm)	
			Mean	S.D.	Mean	S.D.
Ganglion and Ganglion (G-G)	28	0	Not measured ^b		Not measured	
		90	1.3	0.46	1.03	0.43
		180	1.38	0.37	1.10	0.36
		270	1.33	0.48	1.19	0.35
Ganglion and Atrium (G-A)	34	0	1.44	0.40	1.40	0.45
		90	1.4	0.34	1.19	0.34
		180	1.38	0.30	1.18	0.44
		270	1.37	0.34	1.18	0.47
Ganglion and Ganglion (Fused) (G-G)	12	0	Not measured ^b		Not measured ^b	
		90	1.45	0.33	1.10	0.26
		180	1.58	0.41	1.31	0.28
		270	1.46	0.51	1.19	0.33
Ganglion and Atrium (Fused) (G-A)	22	0	1.87	0.77	1.66	0.37
		90	1.57	0.28	1.35	0.36
		180	1.48	0.28	1.35	0.30
		270	1.34	0.28	1.41	0.46
Ganglion and Ganglion (Separate) (G-G)	14	0	0.96	0.51	0.81	0.55
		90	1.23	0.53	0.97	0.56
		180	1.23	0.25	0.94	0.34
		270	1.4	0.46	1.11	0.63
Ganglion and Atrium (Separate) (G-A)	32	0	1.48	0.40	1.22	0.41
		90	1.32	0.33	1.09	0.33
		180	1.37	0.29	1.06	0.35
		270	1.25	0.36	1.0	0.40

^a Because towards the other explant is taken 0°. Other values refer to the clockwise angle (°) against this.

^b In these outgrowth fused between the two ganglia, the outgrowth in this direction could not be measured.

To study directional growth only the general variation of growth has been eliminated by grouping. In each ganglion, the extent of nerve fiber outgrowth as percent of the mean value distance of nerve fiber growth at the four perpendicular directions (Table II). For reasons explained above, the relative values could only be calculated in such ganglion pair starts whose nerve fiber outgrowth did not fuse, while the relative growth data for ganglion and atrium pairs were included whether the growth reached the atrium or not. Had the outgrowth been equidistant to all directions, the mean relative outgrowth values would have been about 100% to all directions. However in ganglion and atrium pair cultures the mean relative outgrowth of nerve fibers towards the atrium (0°) was over 100% in the whole series together in "fused" cultures, and in "separate" cultures whose nerve fiber outgrowth had not reached the other explant or outgrowth from III (Table II). In all 3 instances the difference was statistically highly significant ($P < 0.0001$), testing with the aid of the t -test. To determine the significance of the difference from 100%, a method which best indicates whether or not directional growth had occurred. While the mean outgrowth at 0° increased over 100%,

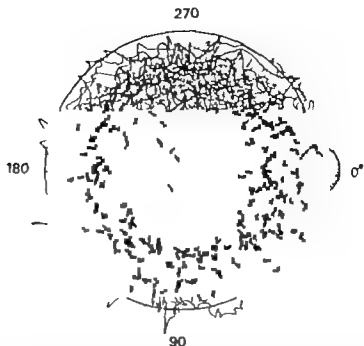


Fig. 3 Outlines of nerve fiber outgrowth around superimposed ganglion explants after culture for 5 days. The ganglion explant is on the left and the atrium explant on the right. A circle has been drawn around the ganglion explant, the distance between the explant centers as a radius. Note the almost round outgrowth pattern around the ganglion explant. The length of nerve fiber outgrowth was measured at the four perpendicular directions indicated by numbers.

(1.64 mm) was larger than that to any of the other 3 directions (1.37–1.42 mm) and this difference was in all instances statistically significant ($P < 0.005$). When measured from the border of explant, essentially similar results were obtained but the mean outgrowth at 0° (1.40 mm) was different from that to 90°–180°–270° (1.18–1.19 mm) at a less significant level ($P < 0.02$).

For those ganglion pair cultures whose outgrowth zones had fused between ganglia (middle third of Table I) the data of outgrowth at 0° were omitted for the same reason as above. A slightly longer mean growth towards the atrium, as compared with growth in other directions, can be seen in those cultures in which the nerve fibers reached the atrium explant.

Data on the "separate" cultures, whose outgrowth did not meet the opposite explant (lower third of Table I) failed to show consistent, statistically significant differences between 0° and the other directions of outgrowth in either pair group. It should be noted that the outgrowths to different directions can only be compared within, not between, the two "separate" culture groups, because all those cultures were excluded from the ganglion pair group whose nerve fibers met between the two ganglia, while only those ganglia were rejected from the ganglion and atrium pair group whose nerve fibers had reached the much longer distance to the atrium explant.

Relative distance of nerve fiber outgrowth

The absolute outgrowth data (mm) given in Table I include the large variations in the general nerve fiber outgrowth from one ganglion to another not only in the direction of growth

area of explant equally applies to the relative outgrowth as measured from the border of explant, which also showed a significant increase in the relative growth towards the atrium and a significant decrease in the relative growth towards another ganglion.

Discussion

Our observations failed to confirm those reported by Chamley *et al.* (1973) and Chamley and Dowel (1975), who found by mere visual examination that many more nerve fibers grow from sympathetic ganglion explants towards normally densely innervated tissues in *in vivo* culture, such as the heart atrium, than away from it or towards explants of such tissues that are normally sparsely innervated. In our cultures, a dense network of nerve fibers rapidly grew to all directions around the explant and visual examination failed to show any distinct tendency of preferential growth to any direction. This equally applied to explants with two ganglia and those with ganglion and atrium explants. No difference was in respect observed between "fused" cultures, whose outgrowth met in the middle of the ganglion explants or reached the atrium explant, and "separate" cultures, in which the outgrowth from the ganglion explant failed to reach the atrium or the outgrowth of the other ganglion.

It is not easy to explain this discrepancy especially since our culture technique was supposedly identical with that employed by Chamley *et al.* (1973) and Chamley and Dowel (1975), the method having been used by one of us (see Eränkö *et al.* 1972 a, b) in the same laboratory where Chamley *et al.* (1973) and Chamley and Dowel (1975) worked. However, there may yet have been small but significant differences. Indeed, while we cultured only 2 explants in each chamber to be able to judge with less ambiguity the eventual growth promoting effect, Chamley *et al.* (1973) and Chamley and Dowel (1975) used 3 rows of explants, 5 ganglion explants in the center row and 2 rows of 5 explants of peripheral organs on both sides of them. Comparison of their photomicrograph (Fig. 4 in Chamley *et al.* 1973) with our (Fig. 1) suggests that the nerve fiber growth around our ganglion explants was much more profuse than that in theirs. If growth of nerve fibers was in their cultures limited for some reason, diffusion of any growth promoting substances from nearby explants can be expected to have a better chance to stimulate it.

Our data were obtained by recording the coordinates on the Vernier scale of the microscope stage and by drawing the outlines of the ganglia and the nerve fiber outgrowth from them. Since nerve fiber outgrowth was always closely accompanied by glial cell outgrowth, there was no difficulty in discrimination between outgrowth of nerve fibers and supporting cells. Monitoring the coordinates of the center of the ganglion explant can be thought to be subject to unconscious bias, and it is therefore important to note that similar results were obtained when measurements were carried out from the border of the explant.

In view of the subjective impression first obtained by mere visual examination that the nerve fiber outgrowth was about the same to all directions, the results of our quantitative measurements were unexpected in showing at a high level of statistical significance that nerve fibers grew farther towards the atrium, as compared with the other directions. This result was obtained for the whole material together as well as for the "fused" and "separate"

TABLE II Relative extent of nerve fiber outgrowth as percent of mean growth towards 4 perpendicular directions in 5 day pair cultures. After culture for 5 days, the distance of outgrowth was measured towards the other explant and towards the 3 other perpendicular directions. In each pair culture the mean of these 4 values was taken as 100% and the outgrowth towards the 4 directions expressed as % of the mean growth, thus equalizing variations between cultures in the per growth.

Paired organs	Number of cultured ganglia	Direction of growth measurement (°) ^a	Relative outgrowth from center of explant (%)		Relative outgrowth from border of explant (%)	
			Mean	S.D.	Mean	S.D.
Ganglion and Atrium (AD)	34	0	112.57 ^b	13.41	113.57 ^b	17.81
		90	98.00	13.12	97.28	16.73
		180	96.06	11.94	95.72	19.46
		270	94.11	13.73	94.15	21.01
Ganglion and Atrium (Fused)	22	0	116.23 ^b	9.42	116.30 ^b	15.40
		90	97.86	10.17	93.55	12.09
		180	92.00	12.28	93.30	17.58
		270	95.86	10.36	96.95	16.12
Ganglion and Ganglion (Separate)	16	0	76.13 ^b	32.24	77.88 ^b	35.66
		90	101.38	35.24	97.56	47.34
		180	111.25	28.97	105.38	31.60
		270	111.38	32.12	119.94	6.71
Ganglion and Atrium (Separate)	32	0	110.06 ^b	15.21	111.56 ^b	11.11
		90	98.09	14.98	99.84	1.11
		180	98.84	11.04	97.25	2.22
		270	92.91	15.68	92.22	2.22

^a Direction towards the other explant is taken as 0. Other values refer to the clockwise angle from this line.

^b This value differs significantly from 100% at the level $P < 0.0001$.

the outgrowth at the other 3 directions correspondingly decreased below 100% and atrium pair cultures, whether the whole material was considered together and "separate" cultures distinctly. However this mean decrease in relative growth direction was not always statistically significant because the difference from 100% at 90°, 180° and 270° was less than that at 0° while the standard deviation the same. This should not detract from the main point that the relative outgrowth to the atrium was significantly larger than that to the other three directions.

"Fused" ganglion and atrium cultures showed a larger relative outgrowth to the atrium (116.23%) than did "separate" cultures of ganglion and atrium (110.06%). This difference was not statistically significant, a highly significant difference between the two groups was observed, when only such cultures were considered in which the distance was 1.5 mm.

In the "separate" ganglion pair cultures the outgrowth towards the other explant was 76.13%, i.e. much less than that to the other 3 directions. This result can also be expressed by saying that the relative distance of growth towards the other ganglion was 76.13% of the mean growth, 100%. This difference was statistically highly significant ($P < 0.0001$).

All what has above been said concerning the relative nerve fiber outgrowth from the

Even, it is too early to speculate on the nature of such postulated substance. Nerve growth factor was proposed by Chamley *et al* (1973) but diffusion of a less specific substance from the strium may have been responsible, many of which are known to have profound effects on the growth and metabolic abilities of nerve cells (Murray 1976, Himes *et al* 1976).

It is even more open to doubt whether release of a specific substance from the target organ is paid to it the nerve fibers from the periphery on in a developing living animal. However the problem is fascinating and continued efforts to solve problems of directional nerve fiber growth can be expected to furnish significant new observations.

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cultures separately whether the measurements were made from the center or the border of the ganglion explant. We conclude therefore that there was a real though small, increase in the mean length of those nerve fibers that grew towards the atrium.

Another unexpected result that emerged from the measurements was the highly significant decrease in the length of nerve fibers growing towards the other ganglion explant in "separate" ganglion pair cultures, as compared with growth to other directions. However, care is necessary in interpreting this observation because the length of growth towards the other ganglion could only be measured in the "separate" ganglion pair cultures whose growth had not fused in the center of the explants, the "fused" ganglion pair cultures having purposely been excluded. It is therefore possible that the shorter mean fiber growth towards the other ganglion was due to selective exclusion of such ganglion pair cultures whose fibers grew better towards the other ganglion. This matter should be re-examined using shorter culture times or a longer distance between the ganglion explants. A similar selection mechanism may have been responsible for the observed tendency towards longer fibers growing towards the atrium in "fused" ganglion-atrium cultures, as compared with "separate" cultures. However, it cannot be excluded that the nerve fibers growing towards the atrium were truly longer because they had contacted the target tissue. Further studies are necessary to elucidate also this point. On the other hand, since the "separate" ganglion-atrium culture group was formed by selecting ganglia whose nerve fibers were short enough not to reach the atrium, it is even more significant that the nerve fibers growing towards the atrium were yet significantly longer than those growing to other directions.

Our main observation is, then, that nerve fibers from the sympathetic ganglion grow towards the atrium were a little, but highly significantly longer than those growing to other directions. The conclusion must be drawn from this observation that the atrium explant somehow stimulates the nerve fiber growth, a conclusion similar to that drawn by Chamk et al (1973) and Chamley and Dowel (1975) as different as their results otherwise were from ours.

The existence of such growth promotion has long been suspected. Since nerve fibers from the proximal stump of a divided nerve tend to enter the peripheral stump, Forssman (1900) proposed that "neurotropism" is responsible, and Ramon y Cajal (1910) thought that nerve fibers might indeed be attracted by chemicals released from tissues. Harrison (1910) formulated the problem as follows: "If it could be shown in tissue culture that there is an attraction between growing nerve fibers taken from a certain part of the nervous system and a particular kind of peripheral cell . . . then we should have direct evidence for the existence of those more subtle factors which seem to be necessary to account for the definitive establishment of particular nervous connections." However, Harrison's (1910) own experiments of this kind gave negative results, and Jacobson (1970) more recently pointed out that "successful experiments of the type that Harrison suggested more than 50 years ago have not yet been made".

In view of these authoritative statements, any evidence of nerve fiber growth stimulation exerted by the target tissue can be considered useful. It seems possible that the atrium explant may have released a substance promoting nerve fiber growth and that a concentration gradient of this substance was formed from the atrium explant to the ganglion explant.

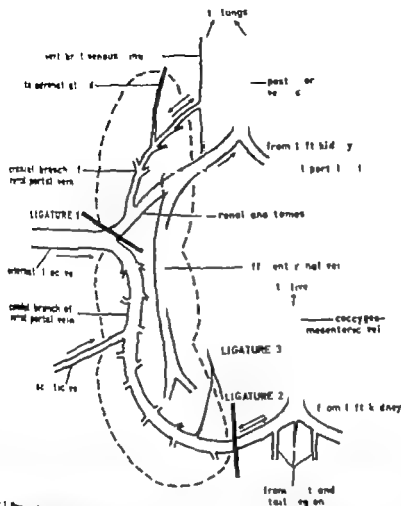


Fig. 1. Proximal veins of the renal portal system of the right kidney seen from the abdominal cavity. Blood from the leg enters the renal portal vein via the external iliac and the sciatic veins, and continues the same pattern flow to the renal portal system of intact legs (Odland 1978). This blood can either bypass the peritubular capillaries of the kidney (bypassing the glomerulus) or be shunted 1 to the lungs to the renal mesenteric and/or the cranial branch of the renal portal vein or 2, to the liver as the coccygeo-mesenteric vein. For further details see Odland (1978). Ligatures 1-3 (see text) are placed around points indicated by cross-marks.

From the foregoing, however, it seems that ideal conditions for use of the Sperber technique could be created if the shunting of renal portal blood could be abolished. It has already been shown (Odland 1978) that, by ligating the appropriate shunt vessels (Fig. 1) all portal blood from the leg can be forced to perfuse the kidney as determined shortly after the operation. In the present study it was tested if this renal perfusion of all portal blood from the leg persisted, so that the true tubular excretion fraction (TTEF Sperber 1948 b), a measure of the tubular excretion efficiency of a substance, could be estimated directly. Since this was found to be the case, it is concluded that excretion studies in ligated animals offer a valuable complement to the original Sperber technique.

A modified Sperber technique for direct estimation of true renal tubular excretion fraction

By

BO ODLIND

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Abstract

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The Sperber technique in the hen is particularly suitable for the study of renal tubular secretion. However, results obtained with this technique vary considerably due to an unpredictable and highly variable shunting of renal portal blood. In an attempt to get a total and stable renal perfusion by portal blood from the leg, appropriate shunt vessels were ligated. This procedure was found to force all portal blood from the leg to perfuse the ligated kidney without affecting the symmetry of glomerular filtration or renal clearance of ^{125}I -Na- α -iodohippurate between the kidneys. In conclusion, the ligation of renal portal shunt vessels allows use of the Sperber technique for a direct estimation of the true tubular excretion fraction (TTEF) of a substance. Thus, TTEF_{PAR} , a measure of the tubular excretion efficiency of para-amino-hippuric acid, was found to be about 70%. The stable renal perfusion in ligated animals will also facilitate comparative studies of renal tubular excretion in the hen. Moreover, no animal has to be rejected due to low renal perfusion of portal blood. Furthermore, the use of ligated hens makes it unnecessary to use markers for the renal perfusion of portal blood when steady-state experiments are performed. Finally, the total renal perfusion of portal blood in ligated animals will facilitate the demonstration of a secretor component in the renal handling of substances with a low affinity for the renal transport system.

Key words: Renal clearance, tubular secretion, kidney birds, microspheres

Based on the renal portal circulation in the hen, Sperber (Sperber 1946, 1948 a, b) has developed a technique, which is particularly suitable for the study of renal tubular secretion, of reviews by Weiner (1973) and Rennick (1976). However, the results obtained with this technique vary considerably due to an unpredictable and highly variable shunting of renal portal blood to the liver and to the lungs (Odlind 1978). In spite of this variation, individual excretion values may be compared, if the fraction of portal blood that perfuses the kidney is known. This fraction can be determined from the tissue distribution of microspheres injected simultaneously with the substance to be tested (Odlind 1978). An alternative method is to co-administer a marker for the renal perfusion, e.g. para-amino-hippuric acid (Rennick 1976). The excretion efficiency of the test substance can then be expressed relative to that of the marker.

Renal tubular excretion fraction of para-aminobenzoic acid (ATEF_{PAH})

para-aminobenzoic acid (PAH) as administered either as 4-5 min bolus injection of tracer doses of NaPAH ($\text{pH} 7.4$, 0.25 mEq/ml, New England Nuclear, USA, 9 hens) or as a continuous infusion (18-25 mg/min/kg of the sodium salt (Merck, Sharp and Dohme Co, USA 14 hens) into a leg vein on the ligated side. After the bolus injections urine was collected for three 10 min periods. The excretion fraction of PAH, its ATEF, as calculated during steady-state as the mean of three consecutive infusions of PAH, its ATEF as determined according to Brum (1951) and 14-C as calculated by liquid scintillation counting ATEF as calculated by dividing the excretion in the same 10 min collection period in each hen. PAH as determined according to Brum (1951) and 14-C as calculated by liquid scintillation counting ATEF as calculated by dividing the excretion in the same 10 min collection period in each hen. The total (ipsilateral plus contralateral) excretion of PAH averaged 92.1 and 96.4% of the given amount after bolus injections and continuous infusion, respectively.

Direct measurement of 14-C-PAH (0.1-10 $\mu\text{g}/\text{ml}$ buffer) to plasma proteins (pooled, heparinized, hen and human plasma) at 37°C as determined by equilibrium dialysis in isotonic phosphate buffer (Pitt, Eberle and Olszko-Czerwik 1973). This is in agreement with the findings by Sykes (1960). S and at Raskin (1976), however found 14% protein binding of PAH in hen plasma. This discrepancy may be due to different properties of the two plasma albumins (McLennan 1962), in the several birds.

*Measurement of the renal clearance of 51-Cr-EDTA (C_{EDTA}) and 125-I-*p*-iodohippurate (C_{IHP})*
To measure better the ligation of vessels in the renal portal system had any effects on the renal function, the clearance of 51-Cr-EDTA (ethylene-diamine-acetra-acetate, Behringwerke AG, West Germany) and 125-I-*p*-iodohippurate (Kabi Diagnostika, Sweden, 17 hens) were determined for the ligated and the control kidneys separately as described earlier (Odland 1978).

Excretion experiments

Each animal was followed by an injection of 57-Co or 58-C⁶⁰ labelled 30 μm microspheres (Tracer label, Pharmacia Fine Chemicals AB, Sweden) into leg veins on the ligated side and sometimes also in the control side, using the distal-branch technique. This technique as described earlier by Odland (1978). The reliable manner of the fractional distribution of blood flow from the external iliac vein.

Statistical analysis

Data are given as the arithmetic means \pm standard deviation (S.D.) or the arithmetic means \pm standard error of the mean (S.E.) when appropriate. Differences in clearance values and ATEF values were analysed statistically with the Mann-Whitney U-test and unpaired observations, respectively (Aronow 1973) and are considered significant at the 0.05 level.

Results

Effect of ligation on the distribution of renal portal blood

After injection of microspheres into a leg vein on the ligated side, nearly all spheres were recovered in the middle and caudal parts of the ligated kidney at 1 1/2-4 h (7 hens) or 2 days (0 hens) after the operation, see Table I. After 4-7 days (19 hens) the recovery was lower than 30% in the same parts of the ligated kidney while the recovery in the lungs was correspondingly higher. These results in ligated animals are in sharp contrast to the highly variable distribution of spheres after leg vein injections in non-ligated animals (Odland 1978). After injection into a leg vein on the control side 4-7 days after the operation, the spheres were distributed between the ipsilateral kidney and the lungs (Table I). No spheres were found in the liver which shows that the shunt flow in the coccygeomesenteric vein from both renal portal systems had been blocked.

Effect of ligation on the venous pressure in the renal portal system

The ligation procedure gave an immediate increase, about 30%, of the pressure in the external iliac vein on the ligated side (Table II). This pressure remained elevated throughout

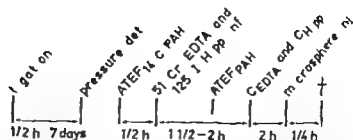


Fig. 2. Experimental schedule. The effects of ligating vessels in the renal portal system was determined in sequence at different times post-operatively using the following parameters. 1 the venous pressure in external iliac veins, 2 the renal excretion of para-aminohippuric acid (ATEF_{PAH}) or 14-C-para-aminohippuric acid (ATEF_{14-O-PAH}), 3 the renal clearances of 51-Cr-EDTA (C_{EDTA}) and 125-I Na-o-iodopurinate (C_{H_{pp}}) and 4 the distribution of 50 μ m microspheres injected into the leg vein.

Materials and Methods

Animals

Laying Rhode Island Red or Kathlin hens, 6-12 months of age and weighing 1.7 to 2.2 kg were used. They were kept on a commercial feed for laying hens (Pullus Lantbruk, Sweden) but were fasted overnight before surgery and clearance experiments. They had free access to tap water except during the experiments.

Experimental procedures

Fig. 2 shows the different steps of the investigation. The animals were always unanesthetized except during the operations. They were loosely restrained in a harness of cloth (Campbell 1960 a) and sitting in its normal perching position, unless stated otherwise. Urine was collected through two plastic funnels set one over each ureteral opening according to a modification (Campbell 1960 a) of Sperber's original technique (1946, 1948 a). To prevent clogging by urinary precipitates, each funnel was perfused with distilled water by an infusion pump at a rate of 1.0 ml/min. The diluted urine was collected in plastic vials on a fraction collector (10 min period), and the volumes determined by weight. Systemic infusions were made into a brachial vein and renal portal infusions (injections) into the right medial tarsal vein, unless stated otherwise. Polyethylene catheters (PP50) were used. Isotonic saline was infused at a rate of 0.15 ml/min throughout the experiments.

Ligation of sham in the renal portal system

Ligatures were placed around the renal anastomosis (ligature 1 in Fig. 1) and the caudal branch of the renal portal vein, where the vessel leaves the kidney (ligature 2 in Fig. 1), as described earlier (Odlind 1977). This was always done on the right kidney and should force all venous blood from the right leg, from the external iliac and sciatic veins, to perfuse the peritubular capillaries of the middle and caudal parts of the kidney. Ideally the same procedure should be performed also on the left side, but here the caudal sham vessel is not accessible to ligation, since it is covered by the dorsal ligament of the oviduct. Instead, an attempt was made to improve the symmetry of total renal blood flow between the kidneys by ligating the coccygeomesenteric vein in its caudal 1/3 (ligature 3 in Fig. 1). This should convey the venous blood from the pelvis, the cloaca and the coprodeum to the left renal portal system. Moreover it should block the shunting of renal portal blood from the left side to the liver. The surgery was performed without special precautions, but no infections were seen in about 50 hens. Acute operation mortality was about 10% mostly due to bleeding. About 5% of the animals were rejected because of complications, e.g. strictures on the right ureter.

Measurement of venous pressure in the renal portal system

In three animals heparinized polyethylene catheters (PP50) were inserted into the medial tarsal vein on both legs, with the tip of the catheters in the external iliac veins. Venous pressure was then determined on both sides using a diaphragm pressure transducer (type 4-327 L221 Bell and Howell Ltd, England) with the unanesthetized animal supine as well as sitting in the normal perching position. Ligation procedures were then performed (see above) and the pressure determinations repeated soon after the operation and at 24 h intervals thereafter. In seven other animals the pressure determinations were done only once, 4-7 days after the operation.

Table II. Renal clearance in ml/min/kg of ^{51}Cr -EDTA (C_{EDTA}) and ^{125}I Na-o-iodohippurate (C_{IHP}) 4-7 days after ligating vessels in the renal portal system (means \pm S.D. calculated from means of 2-3 10 min clearance periods during steady-state in each of 18 and 17 hens, respectively). The paired differences between values for the ligated and the control kidney are also shown (means \pm S.D.).

	C_{EDTA}	C_{IHP}
paired kidney	1.90 \pm 0.33	13.5 \pm 4.3
unpaired kidney	1.53 \pm 0.38	15.5 \pm 5.1
Difference (ligated-control)	0.85 \pm 0.16	-0.02 \pm 2.7

ATEF_{AE} was determined by dividing the ATEF_{AE} value with the renal perfusion fraction of the blood from the leg. It should be noticed, however, that the TEF_{AE} values of non-ligated animals may be too low, since they derive from injections of PAH on the right side, since the mean renal perfusion fraction is about 3/4 of that on the left (control) side (Odland 1978).

Effect of ligatures on renal function

Renal clearance of ^{51}Cr -EDTA (C_{EDTA}) was symmetrical ($p > 0.2$) in ligated animals (Table II). The C_{EDTA} obtained was comparable to that of non-ligated hens of the same lot and determined under similar experimental conditions, 1.43 ± 0.20 ml/min/kg for each side (mean \pm S.D. to 6 hens, unpublished observation). Since C_{EDTA} equals the renal clearance of ^{51}Cr -inulin in the hen (Odland 1976), this indicates that the ligatures had no effect on the glomerular filtration rate. Moreover, the renal clearance of ^{125}I Na-o-iodohippurate was the same for ligated and control kidneys (Table III, $p > 0.9$) indicating symmetry between the kidneys of ligated animals also for this acid which is excreted mainly by tubular secretion. Presumably this indicates symmetry also in total renal plasma flow between the two kidneys.

Discussion

The ligation procedure gave the anticipated results when tested by the microsphere technique shortly and 2 days after the operation, since all spheres were recovered in the middle and the cranial parts of the ligated kidney (Table I). This indicates that the blood from the left leg perfused these parts exclusively. Preventing the leg vein blood from reaching the cranial part of the ligated kidney (Table I) is of little or no importance, since very little of the blood perfuses the same part of the kidney even in intact hens (Odland 1978).

4-7 days after the operation, about 80% of the spheres were recovered in the ligated kidney whereas the rest were recovered in the lungs. In spite of this, similar ATEF_{AE} values were obtained at different times after recovery from the anaesthesia. Moreover, these ATEF_{AE} values were slightly higher than the true tubular excretion fraction of PAH (TEF_{PAH}) of non-ligated animals. This strongly indicates that all portal blood from the left leg perfused the peritubular capillaries of the ligated kidneys also 4-7 days after the ligation. ATEF values obtained in ligated animals thus represent values for the true tubular excretion fraction (TEF). The escape of spheres to the lungs at 4-7 days after the ligation is therefore probably best explained by leakage of some spheres through the peri-

TABLE I Distribution in different organs of 50 μ m microspheres injected into a leg vein at different times after ligating vessels in the renal portal system. Means \pm S.D. or means and range (between brackets) are given.

Organ	Injection side, time after ligation and number of animals			
	Ligated side			Control side
	1 1/2-4 h 7	2 days 7	4-7 days 19	4-7 days 8
Ligated kidney				
Total	98.3 \pm 1.6	95.1 \pm 4.5	80.9 \pm 16.1	39.5 (5.8-81.1)
Cranial part	0.9 \pm 0.5	0.6 \pm 0.4	2.1 \pm 1.6	
Control kidney	0.3 \pm 0.2	0.5 \pm 0.8	0.9 \pm 0.6	0.7 \pm 0.4
Lungs	0.9 \pm 1.0	2.6 \pm 2.6	17.5 \pm 16.2	59.3 (15.2-94.8)
Liver	0.2 \pm 0.1	1.8 \pm 2.8	0.6 \pm 0.5	0.5 \pm 0.3

the whole test period of 7 days. No obvious change was seen on the control side. The pressure fluctuated with the phases of respiration, somewhat less on the ligated side.

Effect of ligatures on the renal excretion of PAH

The apparent tubular excretion fraction of PAH (ATEF_{PAH}) was the same whether determined after bolus injections of 14-C PAH or after continuous infusions of PAH (data not shown). Soon 1 1/2-4 h after the ligation, before the animals had recovered from the pentobarbital anaesthesia, the ATEF_{PAH} values were low. However at 6 h-2 days and 4-7 days after the ligation ATEF_{PAH} were 66.7 \pm 4.3% and 71.6 \pm 1.9% (means \pm S.E. in 6 and 2 hens, respectively; $p > 0.2$). Both values are somewhat higher than the true tubular excretion fractions of PAH (TTEF_{PAH}) 60.1 \pm 1.5% and 65.1 \pm 2.3% in non-ligated hens (Odlind 1978; means \pm S.E. from two consecutive determinations in 9 hens). TTEF_{PAH} (cf. Sperber

TABLE II Venous pressure in cm H₂O in the external iliac veins before and at different times after ligating vessels in the renal portal system in 10 hens. Results are given as means for each side and as means and range for the paired differences between the pressures on the two sides. Asterisks indicate consecutive measurements in each of three animals (see Methods).

Side and body position	Before	Soon after	Time after ligation (days)						
			1	2	3	4	5	6	7
Ligated									
Supine	7.2	9.2	10.2	11.0	14.3	11.3	10.0	12.5	15.8
Sitting	11.5	—	12.3	14.3	19.3	18.8	15.0	18.5	18.8
Control									
Supine	7.3	6.3	6.8	5.3	6.0	4.8	6.0	4.0	5.0
Sitting	11.5	—	9.7	10.3	10.8	13.3	8.3	10.5	11.0
Difference (lig.-contr.)									
Supine	-0.2	2.9	3.3	5.7	8.3	6.5	—	—	10.8
	(-0.5-0)	(2-3.5)	(2.5-4.5)	(3-6)	(3-15.5)	(1-12)			(7.5-14)
Sitting	0	—	2.7	4.0	8.5	5.5	—	—	7.8
			(0-7)	(1-6)	(5-15.5)	(3-9)			(5-10.5)
Number of animals	3	3	3	3	3	6	1	1	2

TABLE II. Renal clearance in ml/min/kg of 51-Cr-EDTA (C_{EDTA}) and 125-I-Na-o-iodohippurate (C_{IHPP}) 4-7 days after ligating vessels in the renal portal system (means \pm S.D. calculated from means of 2-3 10 min clearance periods during steady-state in each of 18 and 17 hens, respectively). The paired differences between values for the ligated and the control kidneys are also shown (means \pm S.D.).

	C_{EDTA}	C_{IHPP}
Left kidney	1.50 ± 0.35	15.5 ± 4.3
Control kidney	1.55 ± 0.38	15.5 ± 5.1
Difference (ligated-control)	0.05 ± 0.16	-0.02 ± 3.7

PAH) was determined by dividing the $ATEF_{PAH}$ value with the renal perfusion fraction of portal blood from the leg. It should be noticed, however, that the $TTEF_{PAH}$ values of non-ligated animals may be too low since they derive from injections of PAH on the right side, since the mean renal perfusion fraction is about 3/4 of that on the left (control) side (Odland 1978).

Effect of ligatures on renal function

The renal clearance of 51-Cr-EDTA (C_{EDTA}) was symmetrical ($p > 0.2$) in ligated animals (Table II). The C_{EDTA} obtained was comparable to that of non-ligated hens of the same lot determined under similar experimental conditions, 1.43 ± 0.20 ml/min/kg for each side (S.D. in 6 hens, unpublished observation). Since C_{EDTA} equals the renal clearance of inulin in the hen (Odland 1976), this indicates that the ligatures had no effect on the glomerular filtration rate. Moreover the renal clearance of 125-I-Na-o-iodohippurate was the same for ligated and control kidneys (Table III, $p = 0.9$) indicating symmetry between kidneys of ligated animals also for this acid which is excreted mainly by tubular secretion. Presumably this indicates symmetry also in total renal plasma flow between the two legs.

Discussion

The ligation procedure gave the anticipated results when tested by the microsphere technique shortly and 2 days after the operation, since all spheres were recovered in the middle and caudal parts of the ligated kidney (Table I). This indicates that the blood from the leg perfused these parts exclusively. Preventing the leg vein blood from reaching the cranial part of the ligated kidney (Table I) is of little or no importance, since very little of the blood perfuses the same part of the kidney even in intact hens (Odland 1978). 4-7 days after the operation, about 80% of the spheres were recovered in the ligated kidney whereas the rest were recovered in the lungs. In spite of this, similar $ATEF_{PAH}$ values were obtained at different times after recovery from the anaesthesia. Moreover these $ATEF_{PAH}$ values were slightly higher than the true tubular excretion fraction of PAH ($TTEF_{PAH}$) of non-ligated animals. This strongly indicates that all portal blood from the leg perfused the peritubular capillaries of the ligated kidneys also 4-7 days after the operation. $ATEF$ values obtained in ligated animals thus represent values for the true tubular excretion fraction ($TTEF$). The escape of spheres to the lungs at 4-7 days after the operation is therefore probably best explained by leakage of some spheres through the peri-

tubular capillaries of the lower 2/3 of the ligated kidney. These capillaries may have been dilated by the persistent increase in renal portal pressure (Table II).

What $ATEF_{PAH}$ value would be expected in non ligated animals? Since the tubular excretion efficiency of PAH is 70% (see Results section), and the mean renal perfusion fraction of portal blood from the leg is 43.5% (range 8–88%), as determined by Odling (1978) using the microsphere technique, a mean $ATEF_{PAH}$ value of 30% (range 6–62%) can be calculated. This predicted value agrees with the mean $ATEF_{PAH}$ value of 30% (range 1–48%) found by Odling (1978). Sykes (1960) and Skadhauge (1964) found a similar average $ATEF_{PAH}$ value. Other workers, however (e.g. Child and Dodds 1965; May and Carter 1970; Acara *et al.* 1975) have reported higher $ATEF_{PAH}$ values, between 50–80%. The reason for this discrepancy is not clear, but the higher values could be due to the preferential use of animals found to excrete a high ipsilateral excess of a test substance, e.g. phenol red, as recommended by Sperber (personal communication) and described by Campbell (1960 b). Moreover the fraction of portal blood that perfuses the kidney on the side of the injection may sometimes be larger than that on the control side (Odling 1978) which would amplify the ATEF value. This latter source of error could explain the extremely high ATEF values (up to 100% sometimes seen (Rennick 1958)).

The incomplete tubular excretion of PAH after one passage through the kidney as indicated by a $TTEF_{PAH}$ value of about 70% could have several explanations. Firstly some renal portal blood could have perfused areas of the kidney that are unable to extract PAH, e.g. the medulla (see review on the renal handling of PAH in the mammalian kidney by Weiner 1973). However Dencker and Odling (unpublished observations) using an autoradiographic technique found that 57-Co labelled 15 μ m microspheres, injected into a leg vein could be seen only in cortical areas of the hen kidney. Moreover a perfusion of the avian medullary tissue by renal portal blood has been considered less likely for anatomical (Sperber 1948 a) and physiological reasons (Skadhauge 1973). Secondly the tubular transport capacity of PAH could have been exceeded, but since the doses of PAH and the tracer doses of 14-C PAH gave equivalent ATEF values in ligated animals, this was certainly not so (cf Odling 1978). Thirdly it is possible that the avian renal handling of PAH also involves tubular reabsorption, as has been demonstrated in *Vecrurus* (Tanner and Kinter 1966) and in several mammalian species (Schnermann and Thureau 1965; Baines *et al.* 1968; Cho and Cafruny 1970). Finally it is possible that there is an incomplete cortical extraction of PAH in the hen, as has been shown to be the case in the cat (Nissen 1967) and in the dog (Aukland and Loyning 1969; Velasquez *et al.* 1972). To evaluate the last two possibilities further studies are needed. Ligated animals offer an advantage in such studies.

An excretion efficiency of PAH after one passage through the avian kidney of about 70% means that the renal clearance of this substance (C_{PAH}) will significantly underestimate the total renal plasma flow in birds. Consequently when C_{PAH} is used to calculate the filtration fraction of the avian kidney the value of 7–8% as reported by Sperber (1960) and Skadhauge (1964) is too high. Pitts (1938) obtained the same value for the filtration fraction using the renal clearance of phenol red. Thus, when trying to estimate the maximum ATEF value that could result from diffusion of a substance through tubular cells into the tubular fluid, as defined by the filtration fraction (Sperber 1948 b), a value below 8% does not exclude

the tubular secretion of the substance (cf Sperber 1948 b Erne and Sperber 1974). Moreover the theoretical limit value for the 'diffusion-ATEF' only applies when all portal blood from the leg perfuses the kidney. This is important, since it has been shown (Odell 1978) that the kidney is perfused by a submaximal fraction of the portal blood from the leg, giving a proportional reduction in the limit ATEF value. Experimental support of this has been obtained in studies of the renal excretion of 125-I iothalamate in the non-ligated hen (Odell 1976). The ATEF value of this substance was 8 %, a figure which would not demonstrate whether the substance is secreted or not. However the substance is indeed secreted by the tubules, since its renal clearance was reduced by half and reached the values of the glomerular filtration rate, as measured by 3-H-inulin or 51-Cr EDTA after inhibiting its active transport system by novobloclin.

It is essential for a correct interpretation of the ATEF values to know if the two kidneys are symmetrical with respect to glomerular filtration and total renal plasma flow (Sperber 1948). Both parameters were symmetrical on an average in ligated animals (Table III) which would justify their use in the Sperber technique, but individual testing for symmetry is still necessary.

Excretion studies in ligated animals offer a valuable complement to the original Sperber technique in several ways. Firstly the true tubular excretion fraction (TTEF Sperber 1948) is a measure of the tubular excretion efficiency of a given substance may be estimated directly. Secondly the large variation in distribution of renal portal blood from the leg system in intact animals (Odell 1978) will be abolished, reducing the intra- and inter-animal variations in the excretion values. This reduces the number of experiments needed for a reliable comparison of the tubular excretion efficiency of different substances. Moreover a normal leg is to be rejected due to a low renal perfusion of portal blood (cf Campbell 1964; Stadhagen 1964; Szener 1965). For the same reasons ligated animals are suitable for steady-state experiments. The stable renal portal blood flow in these animals makes it unnecessary to use markers for the renal perfusion fraction of portal blood from the leg (Szener 1976; Odell 1978 a). Finally the use of ligated animals will facilitate the demonstration of a secretory component in the renal handling of substances with a low affinity for the renal transport system.

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Muscle fatigue and its relation to lactate accumulation and LDH activity in man

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Abstract

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Lactate concentration in different muscle fibre types was determined in biopsy specimens from human vastus lateralis muscle after 30 and 60 s of maximal dynamic leg exercise. In addition, muscle fibre type histogram, total lactate dehydrogenase (LDH) activity and isoenzymes of LDH were determined. In contrast with previous studies (Thorstenson and Karlsson 1976, Nilsson *et al.* 1977) it was found that increased proportion of slow twitch (ST) fibres corresponded to better sustained muscle force. Lactate accumulation was found predominantly in fast twitch (FT) fibres after 30 s, but after 60 s this difference was abolished. Strong correlation between the two muscle fibre types in muscle lactate, total LDH activity and M-LDH activity was observed in muscle fatigue. It was concluded that lactate or associated pH changes mainly in FT fibres could be one factor responsible for the impaired muscle function.

Key words: Fatigue, isoenzymes, lactate, lactate dehydrogenase, muscle fibre types

In a kinetic fatigue test, performed as repeated maximal knee extensions at a high joint velocity was recently carried out in order to study muscle fatigue in human beings (Thorstenson and Karlsson 1976). Fatigability measured as the decline in force with 50 repetitions (approximately 60 s), was found to be closely correlated to the relative contribution of fast twitch (FT or type II) muscle fibres in the recruited muscles. Indeed the fact that slow twitch (ST or type I) muscle fibres are more resistant to fatigue than FT muscle fibres has been well-established from animal experiments (Burke and Edgerton 1975).

In a subsequent series of experiments (Nilsson *et al.* 1977) EMG recordings were included to investigate if the decline in muscle performance was due to factors located in the neuromuscular junction, as indicated by Stephens and Taylor (1972) or in the muscle fibres as suggested by Merton (1954). No changes in the EMG activity with fatigue could be demonstrated and it was therefore concluded that local factors in the muscle fibres (mainly FT) were responsible for the impaired muscle function.

Lactate accumulation in the muscle has often been discussed as one inhibitory factor in

relation to muscle fatigue (Karlsson 1971, Simonson 1971). Tesch *et al.* (1978) has demonstrated a relationship between muscle lactate accumulation and the percentage of fibres after 25 maximal voluntary contractions, using the same experimental set-up as above, indicating a higher lactate production in FT fibres than in ST fibres. Higher activity of the lactate forming enzyme, lactate dehydrogenase (LDH), and a LDH isozyme pattern more in favour of lactate production have also been demonstrated in FT as compared to ST fibres (Sjödin 1976, Thorstensson *et al.* 1977).

The question arises as to whether lactate accumulation changes within recruited muscle fibres are responsible for the impaired muscular function and fatigue. For that reason the present investigation was undertaken to study changes in lactate concentration in the main muscle fibre types during different stages of the fatigue test and to relate these differences to LDH activity and isozyme pattern in the different fibre types.

Subjects, Methods, and Procedure

Nine male physical education students participated in the study. Their mean (\pm S.E.) age, height, weight, $\dot{V}_{O_2 \max}$, the latter determined on bicycle ergometer according to Åstrand and Saltin (1961), were 23 yrs, 179 ± 1 cm, 69.9 ± 1.8 kg, and 4.2 ± 0.1 l min⁻¹ respectively.

Muscle biopsies (Bergström 1962) for histochemical analysis were taken from the vastus lateralis muscle of the left leg at rest. Histochemical staining for myofibrillar ATPase was performed after preincubation at pH 10.3 (Padykula and Herman 1955, Guth and Samaha 1969). Muscle fibres were classified into the two main categories, *i.e.* as fast twitch (FT or type II) fibres or slow twitch (ST or type I) fibres (Eaton 1962). For classification into subgroups FTa and FTb (type II A and type II B), muscle fibre sections were preincubated at pH 4.6 and 4.3 according to Brooke and Kaiser (1970). Muscle fibre area was determined on muscle transverse sections stained for NADH-diaphorase activity (Novikoff *et al.* 1958) based on a method described by Thorstensson (1976). The relative area of FT fibres was calculated according to the formula $(100 \cdot \text{FT/ST area}) / (\text{FT/ST area} + \text{FT} + \text{ST})$.

The subjects performed repeated maximal isokinetic knee extensions with the left leg on 2 different occasions within a week. The angular velocity of their knee extensions corresponded to 3.14 rad s⁻¹ (180° s⁻¹) with a frequency of approximately 50 contractions min⁻¹. This exercise was performed with the subject sitting in a fixed position with his leg attached to the lever arm of an isokinetic apparatus (Cybex II, Lumex Inc., New York, USA). The test procedure and its accuracy and reliability have been described in detail elsewhere (Thorstensson 1976). The first session consisted of 50 muscle contractions. The second session was terminated after 25 contractions. Peak torque in each contraction was recorded continuously.

The highest torque value obtained during the first 5 contractions was defined as initial strength. The mean value for the last 3 contractions in the experiments was compared with initial strength, and both absolute and relative decline in peak torque were calculated.

Muscle biopsies for biochemical analysis were taken from the vastus lateralis with 3–4 s after 30 and 25 knee extensions respectively. The biopsies were frozen in liquid nitrogen and stored at -80°C until analysed. Lactate concentration, total LDH and LDH isozyme activities were analysed on freeze dried and dissected pools of the two main fibre types according to Tesch *et al.* (1978).

Means, standard error of the means (S.E.) and linear regression coefficient (*r*) were calculated. Differences between means were tested for significance using Student's *t* test.

Results

Initial strength averaged 161 Nm (range 117–237). Mean decline in peak torque after 25 contractions amounted to 40 Nm (range 20–75) or 25% (range 16–50) of the initial value. No significant differences between the two experimental occasions were observed for initial

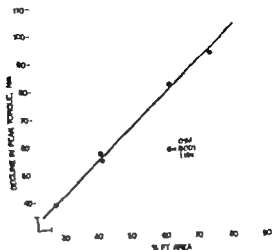


Fig. 1 The relationship between the relative distribution of FT muscle fibres in the vastus lateralis muscle and the decline in peak torque with 50 maximal knee extensions at an isometric velocity of 3.14 rad s^{-1} ($n = 9$).

mean decline in peak torque after 25 contractions. The mean decrease obtained after 50 contractions was 69 Nm (range 39–96) representing a 43% (range 29–59) decline.

Mean muscle fibre type distribution in the present group averaged 48% (range 29–76) FT fibres. FT a and FT b fibre distributions amounted to 29 (range 9–49) and 16% (range 1–39) respectively. Three per cent of the fibres counted were classified as unidentified. The mean cross-sectional area occupied by FT fibres averaged 53 μm^2 (range 28–76).

Both initial strength and decline in peak torque after 50 contractions were positively correlated to the relative distribution of FT fibres, expressed either as the percentage of FT fibres ($r = 0.82$, $p = 0.001$) or as the relative area occupied by FT fibres ($r = 0.94$, $p < 0.001$) (Fig. 1). No correlation was present between torque decline after 25 contractions and muscle fibre type distribution (Table 1). It was not possible to demonstrate any differences in initial strength or decline in peak torque between subjects with a similar muscle fibre type distribution but differing in the FT a/FT b ratio. Thus, the present study did not confirm earlier findings obtained in animal experiments (Burke and Edgerton 1975).

LDH_{act} activity averaged 1.11 (range 0.57 – 1.82) 10^{-4} and 0.61 (range 0.33 – 1.11) 10^{-4} $\text{mmoles min}^{-1} \text{kg}^{-1}$ wet weight in FT and ST fibres respectively. The corresponding values obtained for M-LDH were 0.86 (range 0.57 – 1.76) 10^{-4} and 0.30 (range 0.11 – 0.81) 10^{-4} $\text{mmoles min}^{-1} \text{kg}^{-1}$ wet weight.

The muscle lactate concentration in FT fibres averaged 22.2 (range 4.0–31.0) mmoles kg^{-1} wet weight after 25 contractions and 29.0 (range 11.7–50.5) mmoles kg^{-1} wet weight after 50 contractions. Values obtained in ST fibres were 15.7 (range 3.4–30.5) and 27.0 (range 10.4–53.8) mmoles kg^{-1} wet weight respectively.

A significant difference in lactate concentration was found between FT and ST fibre types after 25 contractions ($p = 0.01$), but this lactate concentration gradient between the two fibre types equalized after 50 contractions (Fig. 2). The FT/ST lactate ratio, i.e. the lactate concentration in FT fibres over the lactate concentration in ST fibres was 1.44 (range 1.18–2.00) after 25 contractions and 1.14 (range 0.88–1.82) after 50 contractions.

relation to muscle fatigue (Karlsson 1971, Simonson 1971). Tessch *et al* (1978) recently demonstrated a relationship between muscle lactate accumulation and the percentage of FT fibres after 25 maximal voluntary contractions, using the same experimental set-up cited above, indicating a higher lactate production in FT fibres than in ST fibres. Higher activity of the lactate forming enzyme, lactate dehydrogenase (LDH) and a LDH isozyme pattern more in favour of lactate production have also been demonstrated in FT as compared to ST fibres (Sjödén 1976, Thorsténsson *et al* 1977).

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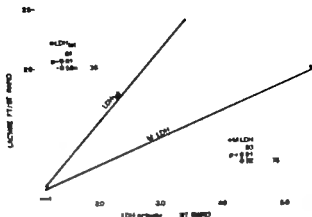


Fig. 1. The relationship between FT/ST lactate ratio, following 15 contractions, and FT/ST ratios for total LDH and M-LDH activity respectively.

muscle fibres (Esmén *et al.* 1975 b, Sjödin 1976). Higher activities of glycolytic enzymes, i.e. LDH and M-LDH (Sjödin 1976, Thorstensson *et al.* 1977), would favour a more rapid lactate formation in FT fibres (Teuch *et al.* 1978). Great differences in the LDH and M-LDH activity between the fibre types, as was found in the present study appear to create conditions for the formation of high lactate gradients between fibre types even if both FT and ST motor units are recruited at onset of exercise (*cf.* Desmedt and Godaux 1977). In addition, there may be a lower rate of lactate release from the FT fibres into the blood stream, since these fibres are normally surrounded by fewer capillaries than ST fibres (Laden 1975).

The lactate concentration of the fibre types had equalized after 50 contractions (approximately 60 s). This may have been due either to a general inhibition of the glycolysis or to a specific inhibition of the myofibrillar enzyme system in the FT fibres (see below). This would in turn result in an increased reliance on ST fibres (which might increase the rate of glycolysis and lactate formation also in this fibre type). In addition, there may be a lactate flow from FT to ST fibres as has been discussed by Esmén *et al.* (1975 a), possibly mediated by membrane-bound M-LDH (Sierjō *et al.* 1968, Sjödin *et al.* 1976). The rate of lactate flow and equalization of the lactate gradient between the fibre types should then be related to M-LDH activity in both FT and ST muscle fibres.

A correlation between muscle lactate concentration and muscle fatigue during 15 min stimulation of frog muscle was recently demonstrated by Flitts and Hoffmeyer (1976). However due to a discrepancy between muscle force and lactate concentration during recovery the authors excluded lactate itself as the direct cause of muscle fatigue. Instead increased pH changes were suggested as one possible factor.

In human skeletal muscle biopsy homogenates good correlations have been demonstrated between lactate concentration and pH (Hermansen and Olesen 1972, Sahlin *et al.* 1976). After heavy exercise pH values in the order of 6.4–6.6 were reported corresponding to lactate concentrations of 25–35 mmol kg⁻¹ wet weight. In the present study mean value for lactate concentration in FT fibres after 15 contractions was 22.2 mmol kg⁻¹ wet weight. It was plausible that similar, or even higher lactate concentrations could be present in certain

TABLE 1 Correlation coefficients (*r*) and levels of significance (*p*) for some variables investigated. (*n* = 9)

	25 contractions		50 contractions	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<i>Force decline¹ versus:</i>				
% FT area	0.59	n.s.	0.94	<0.001
lactate concentration	0.37	n.s.	0.60	n.s.
LDH _{tot} activity ²	0.52	n.s.	0.82	<0.01
M LDH activity ²	0.53	n.s.	0.84	<0.01
lactate concentration, FT/ST	0.71	<0.05	0.30	n.s.
LDH _{tot} activity FT/ST	0.91	<0.001	0.54	n.s.
M LDH activity FT/ST	0.76	<0.05	0.46	n.s.
<i>Lactate concentration, FT/ST versus:</i>				
LDH _{tot} activity FT/ST	0.81	<0.01	0.01	n.s.
M LDH activity FT/ST	0.80	<0.01	0.38	n.s.

Nm. mmol kg⁻¹ wet muscle.

10⁻⁴ mmol min⁻¹ kg⁻¹ wet weight.

This difference was not statistically significant. However, in 8 of the 9 subjects the FT/ST lactate ratio decreased. The muscle lactate FT/ST ratio after 25 contractions was positively correlated both to the corresponding FT/ST ratios for LDH_{tot} activity and M LDH activity (Fig. 3, Table 1). A high lactate ratio was accordingly accompanied by a high LDH and M LDH ratio respectively (Fig. 3). Furthermore, these variables were both correlated to decline in peak torque after 25 contractions (Fig. 4 and 5). Similar relationships were not found after 50 contractions (Table 1).

Discussion

In conformity with earlier studies with the same experimental design (Thorstensson and Karlsson 1976, Nilsson *et al.* 1977), significant correlations were found between the relative incidence of FT muscle fibres, on the one hand, and initial strength and decline in peak torque at a high contraction velocity on the other hand.

The higher lactate concentration in FT fibres observed after the initial 25 rapid dynamic contractions could be due to a primary (Grimby and Hannerz 1968) recruitment of F₁ motor units (Burke and Edgerton 1975) and/or to a higher glycolytic capacity of F₁.

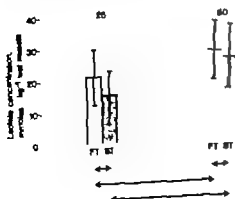


Fig. 4. Lactate concentration in FT (open bars) and ST muscle fibres (shaded bars) after 25 and 50 contractions, respectively. Values are means \pm S.E. (*n* = 9). The number of stars denotes the level of significance (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001) for the differences indicated by the arrows.

lactate in FT fibres. Concomitantly observed differences in the activity of LDH and LDH isozymes between fibre types may influence both the formation of lactate (and H⁺ ions) and the transport of lactate between fibre types, thus indirectly affecting contractile ability and fatigue.

This study was supported by grants from the Swedish Medical Research Council (B77-04X-4251-04A, B78V-421-454) and the Research Council of the Swedish Sports Federation.

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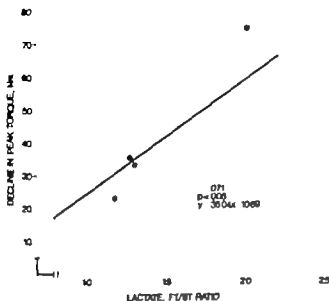


Fig. 4. The relationship between decline in peak torque and FT/ST lactate ratio following 25 contractions.

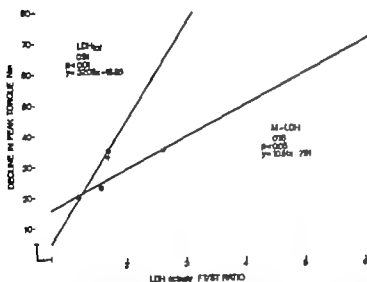


Fig. 5. The relationship between decline in peak torque following 25 contractions and FT/ST ratio for total LDH and M-LDH activity respectively.

extremely fatiguable FT fibres already after 5–10 contractions causing failure to produce tetanus and decline in force either directly or via a decrease in pH.

A mechanism for the high H^+ concentrations to cause impaired muscle function has been demonstrated by Fuchs *et al.* (1970) and Nakamura and Schwarz (1972). They showed that an increase in the H^+ concentration decreases the number of calcium ions bound to troponin during excitation–contraction coupling. This would reduce the number of active actin–myosin interactions, thus decreasing contractile force. Moreover in histochemical studies (e.g. Brooke and Kaber 1970, cf. Methods) it has been observed that the myofibrillar ATPase activity of FT fibres is more sensitive to high H^+ concentrations than that of ST fibres, which also may have functional implications.

To summarize, the present results indicate that initial impairment of muscular function during repeated fast contractions is accompanied by an initial intracellular accumulation of

here in FT fibres. Concomitantly observed differences in the activity of LDH and LDH isozymes between fibre types may influence both the formation of lactate (and H^+ ions) and the transport of lactate between fibre types, thus indirectly affecting contractile ability and fatigue.

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Transient water diuretics and syndrome of inappropriate antidiuretic hormone secretion (SIADH) induced by forebrain lesions of different location

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Abstract

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Lesions of ADH-secretion and transient water diuretics as observed as acute effects of radio-frequency lesions in the septal region of goats. The water diuretics as not compensated for by drinking and therefore acutely induced pronounced hyponatremia and hypovolemia. The development of hypovolemia was accompanied by rise in plasma renin activity. Lesions of the same kind, but extending into the preoptic area near the medial portion of the supraoptic nuclei induced the inability to excrete excessive water diuresis of SIADH. Determinations of plasma arginine vasopressin suggested that the lesions causing SIADH did not produce any noticeable increase in basal ADH-secretion. The results suggest that impulses from post-neuronal receptors replacing ADH-release and water intake to considerable extent are transmitted via the septal region, and that elimination of this impulse traffic is sufficient to turn water intake to the negative side. However reflex volumetric inhibition of the ADH-secretion does not seem to be mediated by pathways passing through the septal region.

A sensitive regulation of antidiuretic hormone (ADH) secretion is of essential importance for the maintenance of normal body fluid composition and volume. According to the present view (cf. Andersson 1977), the main features of this regulation are as follows. The principal factors which determine the rate at which ADH is released from the neurohypophysis are the $[Na^+]$ of the extracellular fluid and the blood volume. ADH-release in response to normal or deviated extracellular $[Na^+]$ is apparently mediated by sodium sensitive receptors which predominantly are located near the anterior wall of the third cerebral ventricle. The volume regulation by all probability consists of both neural and humoral links. Impulses from cardio-vascular distension receptors and the baroreceptors seem to exert a tonic inhibition of ADH-secretion, whereas hypovolemia-induced renal renin release, with subsequent formation of angiotensin II, stimulates ADH-secretion. This effect of angiotensin II appears to be mediated by the same cerebral receptors which are affected by the extracellular $[Na^+]$.

Disturbances in this well balanced regulatory system may lead to an uncontrolled hormone release so-called inappropriate ADH-secretion. The syndrome of inappropriate ADH secretion (SIADH) was first described by Schwartz *et al* (1957) in man. Man is at the only mammalian species in which SIADH is likely to become clinically manifest, since substantial fluid intake, not motivated by thirst, as a rule is the prerequisite for the development of the syndrome. The main characteristics of SIADH are hyponatremia, plasma hypo-osmolality and hypervolemia, together with inability to eliminate with the urine an excessive load of water (*cf* Barter and Schwartz 1967). This syndrome has now and then been observed in connection with CNS disorders in humans (*cf* Barter 1973) and has also been described as an effect of experimentally induced forebrain lesions in the goat (Anderson, Leksell and Lishajko 1975). Another "release phenomenon" concerning water balance observed in that study was the septal polydipsia originally discovered in the rat by Harvath and Hunt (1965).

The intention of the present study was to make a more thorough investigation of SIADH induced by forebrain lesions, and to study whether lesions mainly restricted to the septal region might influence the secretion of ADH.

Methods

Animals. 7 female goats (pre-lesion b.wt. 30–40 kg) were used. The animals were kept in metabolism cages (separating urine from feces) at room temperature ($20 \pm 1^\circ\text{C}$), where they had free access to hay and water. In order to maintain the goats in positive sodium balance, they were given 6 g of NaCl added to a daily ration of 400 g of commercial grain mix. The 4 h water intake and urine output were measured routinely a.m. when samples of the urine were taken for analyses.

Electrode implantation. 5 of the goats had a pair of thermo-couple electrodes (o.d. 0.7 mm) permanently implanted at various levels into the forebrain. The implantations were made under general (nembutal) anaesthesia 2–5 weeks before the electrodes were used for production of radio-frequency (RF) lesions (*cf* Gale 1963). All five animals had the two electrodes bilaterally implanted with an interspace of 3 or 4 mm. The length of the uninsulated ends of the electrodes varied between animals from 3 to 5 mm.

Production of forebrain lesions. Coagulative lesions were made by applying RF energy between the uninsulated ends of the thermo-couple electrodes, having their temperature recording units connected to an Elab thermometer. The tissue temperature between the electrode ends was elevated to 65°C for 5 min. The RF heating was not seen to cause any pain or distress to the animals. Therefore, lesioning was made in the unanaesthetized goat standing in its habitual environment.

Hydration and water supplementation. Hydration was accomplished by giving 75–150 ml/kg of 38°C water by stomach tube into the rumen. Water supplementation was regularly administered in the same manner to one of the goats which developed permanent adipositas as an effect of the forebrain damage.

Routine sampling and analyses. During hydration experiments, and on the day of lesioning, urine was collected via a retention catheter inserted into the urinary bladder. Jugular vein blood samples (10 ml) were drawn into heparinized syringes. For determination of the hematocrit value (Ht) the samples were centrifuged in graded tubes at 3 000 rpm for exactly 7 min. Urine and plasma [Na] and [K] were measured by use of an IL 343⁺ flame photometer and an Advanced Instruments Inc. osmometer was used for determination of the osmolality of these fluids.

Hormone analyses. Ice-chilled syringes with 0.3 M EDTA as anticoagulant were used to obtain blood samples for radioimmunoassays. Determinations of plasma arginine vasopressin (AVP) were performed with a method previously applied for determination of this hormone in human plasma (Fyhrquist, Walenkus and Hollemans 1976 b). Cross-reaction with oxytocin (Sandoz) was $<0.03\%$, with pressorin, acid (Ferring) $<0.01\%$, and with C-terminal tripeptide of vasopressin (Ferring) $<0.01\%$. Sequential saturation and the use of the Sigma VII AVP for standards and preparation of ^{125}I labelled tracer increased the assay sensitivity to 0.2 pg/ml. Recovery of synthetic AVP added to pooled goat plasma was 67–98% in the low range (2.8 pg/ml added), and 82–105% in the high range (28 pg/ml added).

bioassays of plasma renin activity (PRA) were performed with a method modified for goats (Fyfe *et al.* 1974).

Administration of hormones. In order to initiate SIADH, prolonged (50–100 min) infusions of synthetic (17 β -oestrone, Grade V Sigma, 80–360 μ g/kg/min) were made in association with hydration to two goats not used for ablation studies. Hydrocortisone ("Solu-Cortef" Upjohn), was given intravenously to one of the goats which had developed SIADH.

End of experiment. At the termination of the post-lesioning observation period the goats were decapitated under ether anaesthesia and the heads were perfused with isotonic saline followed by 8% formal saline. After brief fixation, block III of the brain (including the preoptic region, the scryum, the thalamus and the hypothalamus) was embedded in celloidene and cut by serial transverse (11 animals), or sagittal (one animal) sections at 30 μ m. The sections were stained either with toluidine blue or according to Loyer (*cf.* Collopy *et al.*).

Values are given as $\bar{x} \pm$ represent mean \pm S.E.

Results

I. Pre-lesioning studies

During the periods between electrode implantation and lesioning (2–5 weeks) the daily water intake varied between animals from 25–50 ml/kg, but remained rather stable in each goat. Urinary water loss was 50–75% of the intake and the range of urine osmolality was 600–1600 mosm/kg. The renal Na⁺ and K⁺ excretion varied between 20–30 and 40–20 mmol/day respectively. Two or three blood samples taken in each goat during the pre-lesioning balance study revealed that the blood plasma composition remained rather stable. The following values ($n=13$) were obtained: [Na⁺] 151.4 \pm 0.6 mmol/l, [K⁺] 4.2 \pm 0.1 mmol/l, osmolality 293 \pm 2 mosm/kg. The Ht remained rather constant (range ± 5) in the single animal, but large interindividual differences were observed (28–39). Unfortunately pre-lesioning plasma AVP and PRA were only determined once in two of the goats (see below).

The effect of hydration (100 ml/kg) was studied once or twice in each animal before lesioning (see Fig. 1). All goats developed positive renal free water clearance (C_{H_2O}) within one hour and maximum water diuresis ($C_{H_2O} = +5.8 \pm 0.6$ ml/min) 1.5 to 2 h after water loading. Blood plasma [Na⁺] was then 148 \pm 1 mmol/l and osmolality 290 \pm 1 mosm/kg. Some increase in renal Na⁺ excretion (<100%) was generally seen during the water diuresis.

II. Effects of forebrain lesioning

A. Transient water diuresis

Within the first post-lesioning hour two of the goats developed positive renal C_{H_2O} . The water diuresis reached maximum level 1 to 2 h later and lasted for 9 and 22 h respectively. Since the diuresis was not compensated for by drinking, the goats lost 3.1 and 3.6 litters of water and became markedly dehydrated as indicated by hypernatraemia (160 and 163 mmol/l), plasma hyperosmolality (325 and 334 mosm/kg) and a 15% increase in Ht. In one of the goats plasma AVP and PRA were determined before, and at 4 and 10 h after lesioning. As shown in Fig. 1 the plasma AVP dropped from 11 to 3 pg/ml as an acute effect of the forebrain damage. The PRA rose from 1.1 to 2.7 ng/ml.h⁻¹ between the 4th and 10th post-lesioning h when the goat had become markedly dehydrated and hypovolemic.

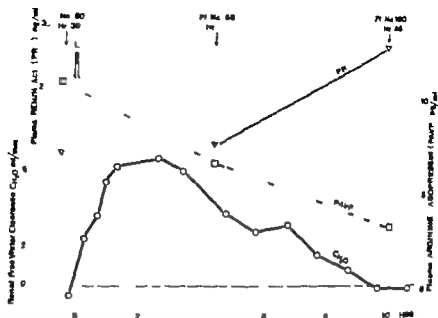


Fig. 1 Inhibition of the ADH-secretion (drop in plasma arginine vasopressin, PVP) and transient water diuresis (positive renal C_{H_2O}) seen as acute effects of radio-frequency lesioning (L) in the septal region. Since the water diuresis is not compensated for by drinking (induces hypernatremia and hypovolemia, indicated by the figures for plasma $[Na^+]$ (in mmol/l) and hematocrit (Ht) on top of the figure. That the developing hypovolemia stimulates the renin-angiotensin system is evidenced by the rise in plasma renin activity (PRA) toward the end of the water diuresis.

One of the goats did not compensate for its water loss by spontaneous drinking within 4 days post lesioning which necessitated temporary water supplementation. It remained hypodipsic during the entire post lesioning observation period (50 days) with constantly elevated plasma $[Na^+]$ (158 ± 1 mmol/l) ($n = 10$). When repeatedly subjected to water loading during this period the goat developed positive renal C_{H_2O} at a plasma $[Na^+]$ of 155 mmol/l and plasma osmolality of 305 mosm/kg. The other goat started to drink again on the day after lesioning but its plasma $[Na^+]$ and osmolality did not return to normal values until 10 days later. At that stage the animal responded to water loading with a water diuresis in an apparently normal manner.

Forebrain lesions The cerebral ablation in common for the goats which developed temporary water diuresis is demonstrated on a midsagittal section and three transverse sections in Fig. 2 (vertically hatched areas). It involved the medial part of the septum and extended just in front of the anterior commissure to a horizontal level passing through that structure.

B Apparent SIADH

Three of the lesioned animals did not display any acute change in renal water excretion, but developed symptoms characteristic of inappropriate ADH secretion. During the post lesioning observation periods (16, 20 and 90 days) the animals were repeatedly ($n = 11$) subjected to water loading (75 to 150 ml/kg). Although in normal water balance when the water load was administered, none of the goats responded with a water diuresis. Instead, hydration

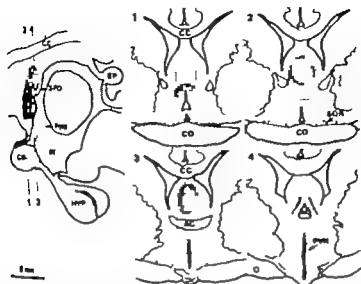


Fig. 2. A sagittal section through the hypothalamic region of the goat (left), and four transverse sections (corresponding to the numbered levels on the sagittal section). The vertically hatched areas represent the brain damage in common for two goats to high RF-lesioning induced transient, uncompensated water diuresis. The horizontally hatched areas show the forebrain damage in common for three goats which developed ADH after lesioning. III - Third cerebral ventricle; AC - Anterior commissure; CC - Corpus callosum; CO - Chiasmatic optic; EP - Epiphysis; HYP - Anterior lobe of the hypothalamus; OT - Optic tract; PVN - Paraventricular nucleus; S - Septal region; SFO - Subfornical organ; SON - Supraoptic nucleus.

induced pronounced hyponatremia, plasma hypo-osmolality, hypervolemia (as indicated by increased Ht), and natriuresis. These effects of hydration are illustrated in Fig. 3.

The glomerular filtration rate (GFR) was not determined. Since reduced GFR, due to deficiency of glucocorticoids, could possibly have contributed to the animals' inability to

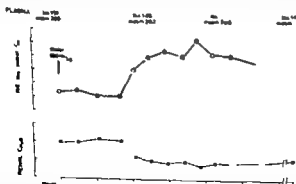


Fig. 3. Apparent inappropriate ADH-secretion (= inability to eliminate with the urine an excessive load of water) in goat 18 days after preoptic lesion of the extent shown in Fig. 2 (horizontal hatching) was noted. Positive renal CaCl_2 does not develop (lower curve) in spite of progressive hyponatremia (as indicated by the values for plasma $[\text{Na}^+]$ in mmol/l) and osmolality in mosmol/kg shown on top of the figure). Note the pronounced increase in renal Na^+ excretion (upper curve) which seems to be an effect of the abnormal expansion of the body fluid volume. Water given by stomach tube into the rumen at zero time.

TABLE I

	Pre-lesion	Post-lesion		
	Non-hydrated	Non-hydrated	Hydrated	
	pl. Na 150.0 mmol/l (n = 1)	pl. Na 150.0 ± 0.8 (n = 8)	pl. Na 140-145 (n = 11)	pl. Na 14 (n = 6)
PAVP (pg/ml)	3.10	3.90 ± 0.68	3.07 ± 0.42	2.82 ± 0.48
PRA (ng/ml.h ⁻¹)	1.19	1.27 ± 0.29	0.43 ± 0.06	0.30 ± 0.04

Plasma arginine vasopressin (PAVP) values, and values for plasma renin activity (PRA) obtained in one of the goats which developed the syndrome of inappropriate ADH secretion (SIADH). The slight reduction AVP seen during post lesion hydration is not statistically significant, whereas the corresponding reduction in PRA is clearly significant ($p < 0.01$). pl. Na = blood plasma [Na].

excrete excessive water 125 mg of hydrocortisone was given intravenously to one of the goats 2 h after the administration of water. In spite of this treatment the renal C_{H_2O} remained negative. However, it seems more likely that the GFR, if anything, increased concomitantly with the hydration-induced expansion of the blood volume. It might explain why the renal Na⁺ excretion reached 3 to 6 times pre-hydration level within 3 h after water loading (Fig. 1).

The reason why the post-lesioning observation periods were relatively short in two of the goats (16 and 70 days) is that in final hydration experiments both animals were given 150 ml/kg of water. Since even this conspicuous hydration did not induce a water diuresis the animals developed symptoms of water intoxication (mild convulsions and signs of pulmonary edema). For ethical reasons the animals were sacrificed as soon as these symptoms became apparent. Half an hour earlier the plasma [Na⁺] had fallen to 120 and 140 mmol/l and the plasma osmolality to 260 and 264 mosm/kg.

AVP and PRA determinations Plasma AVP and PRA were only determined in the goats studied for 90 days after lesioning. Unfortunately, only one determination of the hormones was made during the control period. On that occasion the animal was non-hydrated. Hence the hormonal response to hydration was not studied under normal conditions. However, several determinations of plasma AVP and PRA were made after the brain damage had been induced, both in the non-hydrated and hydrated animal. The results shown in Table I indicate that the forebrain damage did not cause any notable rise in non-hydration AVP or PRA values. After lesioning, hydration seemed to induce a slight fall in plasma AVP but it was statistically insignificant. In contrast, the concomitant fall in PRA was clearly significant ($p < 0.01$).

Thirst defects. The goat in which plasma AVP and PRA were determined became adipsic as a result of its forebrain damage and had to be maintained on daily water supplementation. All experiments made to investigate the animal's response to an excessive load of water were made on occasions when previous water supplementation had restored pre-lesioning water balance (\sim normal b.wt., plasma [Na⁺], osmolality and Ht). The same precautions were undertaken before hydrating one of the other goats which had become hypodipsic after lesioning.



Fig. 4. A transverse section (38 μ m) at the level of the anterior commissure (a-c) showing the lateral and ventral extent of the forebrain lesion in one of the goats which developed SIADH. Note that the ventral border of the lesion on both sides is not far from the medial portion of the supraoptic nucleus (SON). The extent of these nuclei is indicated by dashed lines. The parts of the anterior commissure (a-c) which are not directly affected by the lesion have degenerated. CC = Corpus callosum; CO = Chemical opticon. (See text.)

Forebrain lesions. The main difference in location between the lesions causing temporary water diuresis (see above) and those resulting in SIADH was that the latter extended down into the preoptic region. The brain damage in common for the three "SIADH" goats is shown in Fig. 2 (*horizontally hatched areas*). As seen in transverse section no 2 of this figure, and in Fig. 4, the ventral border of the lesions was not far from the medial portion of the supraoptic nucleus on both sides of the brain. In the adipic goat the cerebral damage extended in posterior direction to involve almost the entire frontal wall of the third ventricle. Only the upper half of the frontal ventricular wall was affected by the lesion in the hypodipic animal.

III. Imitation of SIADH in control animals

Prolonged (50 to 100 min) infusions of synthetic AVP were made in connection with hydration on four occasions in two control goats. The effect on fluid balance was very similar to that observed when the "SIADH" goats were hydrated (*cf* Fig. 3). Thus, when the AVP infusion was started at the same time as water (100 ml/kg) was administered ($n=2$), positive and C_{H_2O} did not develop until 90 min after discontinuation of the infusion. In the meantime, renal Na^+ excretion rose to about 5 times pre-infusion level and considerable hypotension (140 mmol/l) and plasma hypo-osmolality (270 mosm/kg) developed. Likewise, when the AVP infusion was started 1 h after water loading ($n=2$), the resulting, long-lasting inhibition of the water diuresis was accompanied by pronounced natriuresis and hemodilution.

Discussion

A definite proof that the release of ADH from the neurohypophysis is regulated by impulses from the hypothalamus was provided already forty years ago by Fisher, Long and Ranson (1938), who demonstrated that transection of the median eminence caused diabetes insipidus in the cat. Since then, experimental diabetes insipidus has been induced in the same manner in many mammalian species, and it has been shown that the post-renal $\text{C}_{\text{H}_2\text{O}}$ characteristic of diabetes insipidus, might develop within 2 h after RF-coagulation of the median eminence (Olsson 1970). In all these instances, the blockage of the ADH release in non-hydrated animals has been due to interruption of the final neuronal link of the ADH-release mechanism (the axons of neurons in the supraoptic and paraventricular nuclei). However a temporary water diuresis, uncompensated by drinking, was recently observed in two goats as effect of forebrain RF-lesions which did not involve the neurones of the supraoptic and paraventricular nuclei (Andersson *et al.* 1975). In these animals medial placed lesions, which destroyed most of the anterior wall of the third ventricle, also caused permanent adipala. It was suggested that the brain damage had inactivated cerebral sensory mechanism regulating water intake and ADH-secretion, but had left intact reflex volume regulation of the ADH-release in gear. Unexpectedly a similar acute post-lesioning water diuresis was observed here as effect of damage mainly confined to the septal region. Determinations of plasma AVP made in one of the animals (Fig. 1) revealed that the water diuresis was in fact due to a retardation of the basic ADH release occurring in non-hydrated, but water replete animals. The brain damage in these goats obviously also interrupted a regulation of water intake dependent upon extracellular $[\text{Na}^+]$ and circulating angiotensin II. Thus, the excessive acute water loss was not compensated for by drinking in spite of a progressive rise in plasma $[\text{Na}^+]$ and hypovolemia induced activation of the renin-angiotensin system (indicated by the increase in PRA shown in Fig. 1). Since the cerebral damage in these animals did not include the anterior wall of the third ventricle, it appears unlikely that juxtaventricular receptors involved in the control of water balance (*cf.* Andersson 1977) were destroyed to any appreciable extent. More likely is that the septal lesions interrupted pathways transmitting stimulatory impulses from juxtaventricular receptors to the supraoptico-neurohypophyseal system. Only afferents from receptors located near the antero-dorsal wall of the third ventricle or further posterior along the cerebroventricular system seem to have been involved since the forebrain damage did not extend below the level of the anterior commissure (Fig. 2). However receptors regulating ADH-release and water intake are apparently present also near the ventral portion of the third ventricle, *i.e.* below the level of the anterior commissure (*cf.* Andersson 1977). Therefore, the effects of septal lesions observed in the present study indicate that elimination of afferent impulses from only a certain proportion of the juxtaventricular receptor population is sufficient to turn water balance to the negative side.

20 years ago Walker (1957) suggested that the apparent inappropriate secretion of ADH seen in hydrated subjects during anaesthesia is due to removal of a tonic inhibition of spontaneous intrinsic activity in the supraoptic neurohypophyseal system. That autonomous activity may be present in this final neuronal link of the ADH-release mechanism has later been indicated by electrophysiological recording of discharges in supraoptic nuclei isolated

in the rest of the brain (Soda, Koizumi and Brooks 1963). It appears likely that cardiovascular detection and pressor receptors (cf Gauer Henry and Behn 1970) may exert a stimulation of the ADH-secretion in the manner originally suggested by Walker (1957). Inhibition of this kind of tonic inhibition was suggested to have caused the apparent inappropriate ADH-release previously observed as effect of forebrain lesions encroaching on the supraoptic nuclei in the goat (Anderson *et al.* 1975). It also seems to be a plausible explanation of the SIADH observed in the present study which provides some additional information on the matter. The fact that extensive septal lesions as such, did not impair the ability to eliminate excessive water implies that reflex volumetric inhibition of the ADH release predominantly is mediated by pathways not passing above the anterior commissure.

The plasma AVP determinations made in one of the "SIADH" goats do not allow any far-reaching conclusions, since only one AVP value was obtained before lesioning. These few data are in accordance with the previous observation in man (Miller and Moses 1972) that SIADH may be present without any noticeable increase in the basic ADH-secretion. Further studies are, however, obviously needed to disclose whether this is characteristic of SIADH induced by forebrain lesions.

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The distensibility of the resistance vessels of skeletal muscle in hypertensive patients

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Abstract

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The distensibility of the resistance vessels of skeletal muscle was evaluated in 23 patients with essential arterial hypertension (WHO grade 1 II), and 11 normotensive control subjects. Five of the hypertensive patients were well treated for over 2 years and 18 were untreated. The ^{133}Xe wash-out rate from a terfor tibial muscle during reactive hyperemia was recorded before and during an increase of the vascular transmural pressure brought about by application of a subatmospheric pressure to the leg. At ambient pressure the ^{133}Xe wash-out rate did not differ between the normotensive and hypertensive subjects reflecting equal muscle blood flows. However when the transmural pressure was augmented the ^{133}Xe wash-out rate in the normotensive subjects increased about twice as much as in the hypertensive patients. This suggests a decreased distensibility of the resistance vessels in hypertensive patients as compared to normotensive subjects, and supports the concept that structural changes of these vessels take place in arterial hypertension. Since the vascular distensibility was equally reduced in the untreated and treated hypertensive patients it is indicated that the structural changes in the resistance vessels of the leg muscles do not readily decline during antihypertensive treatment.

Key words: Arterial hypertension, muscle blood flow, reactive hyperemia, vascular compliance, vascular transmural pressure, ^{133}Xe wash-out technique.

Chronic arterial hypertension seems to be associated with a reduced vascular distensibility. In animals with arterial hypertension *in vivo* and *in vitro* studies have demonstrated a decreased distensibility (or compliance) of the aorta, the femoral artery and the resistance vessels (Feigl *et al* 1963, Aars 1968, Hallböök *et al* 1974). Although the decreased distensibility apparently applies to the entire arterial system the changes of the resistance vessels are undoubtedly of primary importance from a hemodynamic point of view as the major pressure drop from the aorta to the central veins occurs here.

In hypertensive human subjects *in vitro* studies have shown a decreased distensibility (or increased stiffness) of the aorta and the carotid sinus (Hallock and Benson 1937).

Auerbach and Kreuziger 1951). Moreover a study of the exposed brachial artery has demonstrated a smaller *in vivo* distensibility of this artery in hypertensive than in normotensive subjects (Green *et al.* 1966).

An investigation of the distensibility of the resistance vessels in arterial hypertension has so far not been undertaken in man. A main difficulty of evaluating the true distensibility of the resistance vessels *in vivo* is avoiding reflex constriction of vascular smooth muscles when the distending pressure is augmented. However during reactive hyperemia after even ischemic exercise, the resistance vessels behave like passive-elastic tubes to changes in the vascular transmural pressure, suggesting that all vasoconstrictor activity is abolished (Harting *et al.* 1976, Neuhauer 1977). Thus, in this situation it seems possible to evaluate the inherent elastic properties of these vessels.

In the present study the distensibility of the resistance vessels in the anterior tibial muscle was evaluated in untreated and well treated hypertensive patients, as well as in normotensive subjects. This was accomplished by recording the increase in regional muscle blood flow caused by an increase in the vascular transmural pressure during reactive hyperemia.

Material and methods

The study comprised 18 patients (12 males and 6 females) with essential hypertension (WHO grade I-II (WHO 1962)) receiving no antihypertensive treatment, 5 patients (4 males and 1 female) in the same state of disease but carefully treated with adrenergic beta-receptor blocking agents and diuretics for more than 10 years, and 14 healthy normotensive control subjects (11 males and 3 females). The age, height, and weight distribution was approximately the same in the three groups. (Mean age 41 years; range 27-60 years.)

All of the subjects consented to participate in the study after being informed in detail of its nature and purpose.

The patients were all from the out-patient clinic. The clinical examinations included an X-ray study of the heart, ECG, ophthalmoscopic examination of the eye fundus, test for proteinuria, urine microscopical examination, wash-out urography or radioisotope renography and determination of plasma creatinine and electrolytes.

In each subject peripheral pulses were palpated (a dors ped., and tib. post.), and the systolic blood pressure at the level of the ankle and the first toe were measured by mercury-in-manila strain gauge sphygmomanometry in order to ensure that no hemodynamic significant obstructive arterial disease of the lower extremities was present. In the supine body position the blood pressure at ankle level should not be lower than the systolic blood pressure at heart level measured sphygmomanometrically on the upper arm and the blood pressure of the first toe should not be lower than 30 mmHg below the systolic blood pressure (Nelson *et al.* 1972).

The post ischemic blood flow of the anterior tibial muscle was evaluated by means of the ^{125}I -Xenon wash-out technique (Lassen *et al.* 1964, Harting *et al.* 1976). The subject was supine with the lower leg supported by cushions so that the tibial muscle was at heart level. Approximately 0.2 ml of sterile aqueous sodium chondroitine ^{125}I -Xenon (0.5-1 mCi/ml) was injected into the anterior tibial muscle. Ischemia of the lower leg was induced by means of an inflatable cuff (8.5 cm wide) placed around the distal part above the knee and inflated to supra-systolic pressure (Fig. 1). After 3 min the subject performed isometric exercise (rhythmic dors-plantar flexions of the unloaded foot) until severe pain made further movements impossible. Then the lower leg was enclosed in a rigid PVC tank that fitted airtight just above the knee without compression of the skin (Fig. 1). After deflation of the cuff the ^{125}I -Xenon wash-out curve was followed at ambient pressure for about 1 min. Then the pressure in the tank was lowered to 20 mmHg below atmospheric within 1-2 min and the effect on the wash-out curve observed for about 1 min (Fig. 1). For each subject the areas of the values obtained from the two legs was calculated.

During the period of reactive hyperemia the ^{125}I -Xenon wash-out curve was continuously recorded by a scintillation detector mounted about 20 cm from the leg and pointing at the area of the isotope deposit (Fig. 1). The detector was coupled to channel analyzer, logarithmic recording potentiometer and to a

The distensibility of the resistance vessels of skeletal muscle in hypertensive patients

By

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Abstract

HARTLING O T L SVENDSEN, P E NIELSEN and J TRAP-JENSEN. *The distensibility of the resistance vessels of skeletal muscle in hypertensive patients* Acta physiol. scand. 1978, 103 430-436

The distensibility of the resistance vessels of skeletal muscle was evaluated in 23 patients with essential arterial hypertension (WHO grade I II), and 11 normotensive control subjects. Five of the hypertensive patients were well treated for over 2 years and 18 were untreated. The ^{133}Xe wash-out rate from the anterior tibial muscle during reactive hyperemia was recorded before and during an increase of the vascular transmural pressure, brought about by application of a subatmospheric pressure to the leg. At ambient pressure the ^{133}Xe wash-out rate did not differ between the normotensive and hypertensive subjects reflecting equal muscle blood flows. However when the transmural pressure was augmented the ^{133}Xe wash-out rate in the normotensive subjects increased about twice as much as in the hypertensive patients. This suggests a decreased distensibility of the resistance vessels in hypertensive patients as compared to normotensive subjects, and supports the concept that structural changes of these vessels take place in arterial hypertension. Since the vascular distensibility was equally reduced in the untreated and well treated hypertensive patients it is indicated that the structural changes in the resistance vessels of the leg muscles do not readily decline during antihypertensive treatment.

Key words: Arterial hypertension, muscle blood flow reactive hyperemia, vascular compliance, vascular transmural pressure, ^{133}Xe wash-out technique

Chronic arterial hypertension seems to be associated with a reduced vascular distensibility. In animals with arterial hypertension *in vivo* and *in vitro* studies have demonstrated a decreased distensibility (or compliance) of the aorta, the femoral artery and the resistance vessels (Feigl *et al* 1963 Aagaard 1968, Hallböök *et al* 1974). Although the decreased distensibility apparently applies to the entire arterial system the changes of the resistance vessels are undoubtedly of primary importance from a hemodynamic point of view as the major pressure drop from the aorta to the central veins occurs here.

In hypertensive human subjects *in vitro* studies have shown a decreased distensibility (or increased stiffness) of the aorta and the carotid sinus (Hallock and Benson 1937

TABLE I. Arterial mean blood pressure, ^{133}Xe wash-out rate (k-values) from the anterior tibial muscle, and calculated "vascular resistance" (mean blood pressure/k-value) during reactive hyperemia in normotensive and hypertensive subjects. Changes induced by exposing the leg to vasodilator pressure of 30 mmHg. Mean values \pm one standard deviation (S.D.) are presented.

		Mean blood pressure (mmHg)	^{133}Xe wash-out rate k-value (min^{-1})	"Vascular resistance" (mean blood pressure/k-value) (mmHg-min)
Normotensive subjects (n=11)	mean	106 ± 11.1	0.90 ± 0.27	120.4 ± 29.3
	change	-1 ± 0.8	$+0.34 \pm 0.12$	-38.0 ± 19.4
	per cent change		$+41.2 \pm 18.1$	-28.3 ± 8.9
Hypertensive subjects (normotensive) (n=12)	mean	136 ± 16.7	0.95 ± 0.18	144.3 ± 31.9
	change	-2 ± 4.1	$+0.16 \pm 0.11$	$-22.0^* \pm 13.6$
	per cent change		$+17.3 \pm 13.2$	-15.4 ± 9.4
Hypertensive subjects (hypertensive) (n=9)	mean	109 ± 8.7	0.90 ± 0.30	129.1 ± 38.7
	change	-2 ± 2.4	0.14 ± 0.09	$21.0^* \pm 19.6$
	per cent change		$+16.7^* \pm 14.9$	-16.1 ± 10.0

*Significant difference between normotensive and hypertensive subjects, $P < 0.05$.

†Significant difference between normotensive and hypertensive subjects, $P < 0.01$.

Discussion

The study demonstrates that the ^{133}Xe wash-out rates (k-values) from the anterior tibia muscle during reactive hyperemia at ambient pressure are essentially the same in normotensive and hypertensive subjects. This indicates equal blood flows in normotensive and hypertensive subjects, but higher vascular resistance in the untreated hypertensive patients in proportion to the elevated blood pressure. These results are in good agreement with those obtained by Amery *et al.* (1969) using the same technique.

The main result of the present study is, however, that patients with hypertension respond to a given increase in vascular transmural pressure (distending pressure) with a smaller increase in the ^{133}Xe wash-out rate (Δk)—absolute and fractional—than do normal subjects. This reflects a smaller increase in the muscle blood flow. Since the variation in blood flow predominantly depends on the change in luminal diameter of the resistance vessels the finding of a smaller flow increase in response to increased transmural pressure suggests a reduced distensibility or compliance of these vessels in hypertension.

Evidently it is presupposed that the experimental situation (local anoxia created by the combined effects of arrested blood flow and muscular exercise), caused complete relaxation of the smooth muscle cells in the resistance vessels. Thus it is assumed that arteriolar constriction—which would normally be elicited by an increase in the vascular transmural pressure (cf. Henriksen, 1977)—was abolished by unresponsiveness of the vascular smooth muscle. Previous studies indicate that complete vascular relaxation can indeed be attained during reactive hyperemia both in normal and hypertensive subjects (Folkow *et al.* 1958, Conway 1963, Severinghaus 1970). Thus it has been shown that prolongation of the ischemic period or intraarterial infusion of the potent vasodilating substance, adenosinetriphosphate, do not further augment the muscle blood flow (Folkow *et al.* 1958, Conway 1963). Moreover

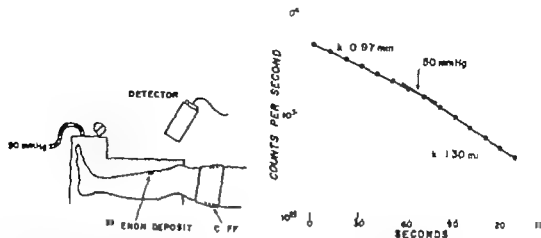


Fig. 1 *Left part* Diagram of the experimental setup. *Right part* Typical ^{231}Xe wash-out curve from the anterior tibial muscle during reactive hyperemia in a normotensive subject before and during application of a subatmospheric pressure to the leg.

digital printer. The half-time value ($t_{1/2}$) was read from the wash-out curve, and the ^{231}Xe wash-out is constant, the k -value in min^{-1} calculated as $k = \ln 2/t_{1/2}$.

Statistical evaluation of the data was made by means of the Student's t -test for paired and unpaired observations, and by means of correlation coefficient analysis. Differences were considered to be significant if a P -value below 0.05 was obtained.

Results

At ambient pressure the ^{231}Xe wash-out rates (k values in min^{-1}), during reactive hyperemia of the treated and untreated hypertensive subjects did not differ significantly from those of the normotensive subjects (Table I). As a corollary of the elevated blood pressure, estimated vascular resistance was higher in the untreated hypertensive subjects. In the small group of well treated hypertensive patients vascular resistance was lower than that of the untreated patients, but the difference was not statistically significant (Table I).

The ^{231}Xe wash-out rate increased significantly ($p < 0.01$) both in normal subjects and in hypertensive subjects when the vascular transmural pressure was augmented by application of a subatmospheric pressure of -50 mmHg to the leg (Table I, Fig. 2). However, this increase in the k value (Δk) was much greater in the normotensive subjects than in both untreated and well treated hypertensive patients, there being no difference of the Δk between the two hypertensive groups (Table I). Correspondingly the fall in estimated vascular resistance was far more pronounced in the normal subjects. There was a small but significant negative correlation between the arterial mean blood pressure and the increase in the ^{231}Xe wash-out rate when the untreated patients and the normal subjects were taken together ($r = -0.55$ estimating equation $y = -0.004x + 0.71$ with y signifying Δk (min^{-1}) and x arterial mean blood pressure (mmHg)).

Table I indicates that the arterial blood pressure in both normotensive and hypertensive subjects remained largely constant during the ^{231}Xe wash-out measurements. However, the blood pressures measured during the examination were 5–6% above the pre-examination levels in all groups, no doubt due to the unfamiliar experimental situation and the ischemic pain.

to the maintenance of the hypertensive state (cf Folkow *et al* 1973). Therefore it is an important question whether these changes are reversible or not. Studies of hypertensive man have clearly shown that the vascular changes can be largely prevented by antihypertensive treatment provided this is instituted at an early age. If on the other hand, the animal is not treated until it is middle-aged the changes are only partially reversible (Webs 1974). In arterial hypertension in man vascular structural changes of the hand, indicated by lowered blood flow resistance at maximal dilatation, were reported by Shertson (1977) to be largely reversible after five years antihypertensive treatment. In contrast similar changes of the calf muscles did not decrease significantly after six months treatment. In the present study the vascular distensibility of the resistance vessels in the anterior tibial muscle was equally reduced in untreated hypertensive patients and in patients having been well treated in the course of at least two years. Although the solution of this problem requires first a larger group of hypertensive subjects receiving effective antihypertensive treatment it is noted, the previous and present findings indicate that the vascular structural changes of the leg muscle do not readily decline despite appropriate antihypertensive treatment. The possible difference in reversibility of the vascular changes in the upper and lower limbs might be explained by the different vascular transmural pressures in these two zones during active hours. Except when the supine body position is assumed the transmural pressure in the legs is higher than in the arms due to the higher hydrostatic pressure. Thus when the systemic blood pressure is reduced through antihypertensive treatment the relative decrease in the vascular transmural pressure will be smaller in the legs than in the arms.

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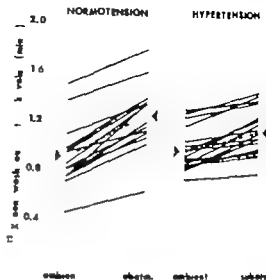


Fig. 2. The effect of exposing the leg to a subatmospheric pressure (subatm.) on the ^{133}Xe wash-out rate from the anterior tibial muscle during reactive hyperemia in normotensive and untreated hypertensive subjects. The mean changes are indicated by the stippled lines.

during reactive hyperemia the resistance vessels are insensitive to vasoconstrictor drugs (e.g. noradrenaline, angiotensine), even in high doses (Sivertsen 1970).

It might be argued that the difference in Δk between normotensive and hypertensive subjects could be explained by the very relationship between the vascular transmural pressure and the blood flow (cf. Folkow and Löfving 1956). The curve formed by this relationship is concave to the pressure axis so that the increase in flow for a given increase in transmural pressure is smaller the higher the initial transmural pressure is. And as the transmural pressure is raised in hypertension because of the larger pressure head, a smaller flow increase could be expected. However, this explanation does not satisfy the present findings since the increase in flow (Δk) was equally small in untreated and in well treated hypertensive patients, even though the pressure head of the latter was not different from that of the normotensive subjects. Moreover, Hallböök *et al.* (1974) in perfusion studies on the hindquarters of normotensive and spontaneously hypertensive rats demonstrated that the resistance vessels of the hypertensive rats were always less distensible than those of the normotensive rats, even when the internal vascular radii and/or transmural pressures were initially the same in the two groups.

The finding that the resistance vessels exhibit a decreased response to stretching forces, in a situation where their vascular smooth muscle cells can be assumed to be completely relaxed strongly indicates that the very structure of the vascular wall is changed in arterial hypertension. This was first suggested by Folkow and coworkers (1958), who demonstrated that in hypertensive subjects an increased thickness of the vessel wall in relation to the lumen might by itself account for the hyperactivity to constrictor agents without necessarily implying an increased activity of the smooth muscle cells. This concept is further supported by later hemodynamic (Conway 1963, Folkow *et al.* 1970, Sivertsen 1970, Folkow *et al.* 1973) and morphological (Short 1966, Suwa and Takahashi 1971) investigations in man and animal.

The vascular structural changes may be adaptive in nature but will probably contribute

Observations on stretch reflexes in lumbar back muscles of the cat

By

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Abstract

CARLSON, H. Observations on stretch reflexes in lumbar back muscles of the cat. *Acta physiol. scand.* 1978. 103, 437-445.

Responses to brief muscle stretch and to electrical stimulation of dorsal roots were studied in cat *li-parses* and ilio-costalis muscles by kymographic and electromyographic recordings in spinal and isolated preparations. Brief stretch applied simultaneously to both muscles, by pulling at an isolated segment of the iliac bone, elicited contractions only in the central region of longissimus, composed of finely anastomosing fibers, the time to peak tension varying between 70 and 100 ms. No reflex responses were observed in the faster contracting parts of this muscle or in the ilio-costalis under the stimulation methods used. The total reflex time usually varied from 4.5 to 7 ms which is shown to correspond to intraspike conduction times, approximately between 2 and 5 ms. Only exceptionally was a central reflex delay corresponding to monosynaptic transmission observed. Reflexes evoked by dorsal root stimulation (L_5 , L_6) had central conduction times similar to those elicited by adequate stimulation. The possibility that the neural pathway for stretch reflexes in longissimus may involve more than two neurons is considered. In isolated preparations the central region of longissimus displays a prominent tonic stretch reflex. With its magnitude as well as the magnitude of the reflex is highly dependent on the position of the lumbar spine relative to the pelvic girdle.

The reflex response of an extremity muscle evoked by a brief muscle stretch, the myotatic reflex, is mediated through a two-neuron arc in which group I fibers originating from the muscle spindles form the afferent component of the pathway (for review see Matthews 1972). The type of proprioceptive reflex control is missing in certain muscles, as e.g. the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The two former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

At the spinal level, absence of monosynaptic reflexes in the dorsal muscles of the cat neck has been reported by Abrahams, Richmond and Rose (1975) even though the muscles are rich in spindles and the motoneurons receive monosynaptic excitation from low-threshold homonymous muscle afferents (Anderson 1977). However the reports give no information as to whether or not the dorsal neck muscles contract in response to adequate stimulation, i.e. stretch.

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Responses to brief muscle stretch and to electrical stimulation of dorsal roots were studied in cat longissimus and iliocostalis muscles by myoelectric and electromyographic recordings in spinal and anaesthetized preparations. Brief stretch applied unilaterally to both muscles, by pulling at an isolated spot of the iliac bone, elicited contractions only in the central region of longissimus, composed of fast conducting fibres, the time to peak tension varying between 70 and 100 ms. No reflex responses were observed in the faster contracting parts of this muscle or in the iliocostalis under the stimulation regimen used. The total reflex time usually varied from 4.5 to 7 ms which is shown to correspond to intrajoint reflexes. The total reflex time usually varied from 2 and 5 ms. Only exceptionally was central reflex delay exceeding the monosynaptic transmission observed. Reflexes evoked by dorsal root stimulation (L_6 , L_7) are usual reflexes time similar to those elicited by adequate stimulation. The possibility that the fast pathway for stretch reflexes in longissimus may involve more than two neurones is considered. I conclude from the present study that the central region of longissimus displays a prominent tonic stretch reflex. With the present method the magnitude of the reflex is highly dependent on the position of the lumbar spine due to the pelvic profile.

In the response of an extremity muscle evoked by a brief muscle stretch, the myoelectric reflex is mediated through a two-neuron arc in which group I fibers originating from the muscle spindles form the afferent component of the pathway (for review see Matthews 1972). In type of proprioceptive reflex control is missing in certain muscles, as e.g. the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

In the spinal level, absence of monosynaptic reflexes in the dorsal muscles of the cat has been reported by Abraham, Richmond and Rose (1975) even though the muscles are rich in spindles and the motoneurons receive monosynaptic excitation from low-threshold homonymous muscle afferents (Anderson 1977). However the reports give no information as to whether or not the dorsal neck muscles contract in response to adequate stimulation, i.e. stretch.

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Reflex responses to brief muscle stretch and to electrical stimulation of dorsal roots were studied in cat hip adductors and abductors muscles by myographic and electromyographic recordings in spinal and encephalic preparations. Brief stretch applied simultaneously to both muscles, by pulling at an isolated point of the skin base, elicited contractions only in the central region of longissimus, composed of slowly contracting fibers, the time to peak tension varying between 70 and 100 ms. No reflex responses were observed in the faster contracting parts of this muscle or in the iliocostalis under the stimulation conditions used. The stretch reflex time usually varied from 4.5 to 7 ms, which is shown to correspond to intraspinal excitation times, approximately between 2 and 5 ms. Only exceptionally was a central reflex delay corresponding to monosynaptic transmission observed. Reflexes evoked by dorsal root stimulation (L_5 , L_6) had central conduction times similar to those elicited by adequate stimulation. The possibility that the neural pathway for stretch reflexes in longissimus may involve more than two neurons is considered. In lumbar preparations the central region of longissimus displays a pronounced tonic stretch reflex. With its help it is seen that the magnitude of the reflex is highly dependent on the position of the lumbar spine and on the pelvic girdle.

The reflex response of an extremity muscle evoked by a brief muscle stretch, the myotatic reflex, is mediated through a two-neuron arc in which group I fibers originating from the muscle spindles form the afferent component of the pathway (for review see Matthews 1972). The type of proprioceptive reflex control is missing in certain muscles, as e.g. the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The two former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

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Thus, it is obvious that the presence of muscle spindles does not necessarily ensure a monosynaptic reflex activation of a muscle. A recent study of the cat's dorsal muscles in the lumbar region has shown that the muscles are supplied with spindles (Carlson 1978 b) but no evidence is available pointing to a monosynaptic transmission of reflexes in the longissimus and iliocostalis (cf Carlson and Lindquist 1976). These two muscles form the main portion (about 80%) of the muscle mass in the lumbar back region, and since their contraction time is short (about 30 ms) they are well suited for quick phasic action. However in a particular region of the longissimus there is a large proportion of slowly contracting fibers (contraction time about 100 ms) that can be selectively activated in reflexes evoked by dorsal root stimulation (Carlson 1978 a, b).

In the present investigation a closer analysis has been performed of reflex responses set up in the longissimus and iliocostalis muscles on application of adequate stimulation by brief and tonic stretch. The role of the longissimus in the control of the axial skeleton has also been analyzed.

Material and Methods

Preparations: Experiments were performed on 15 adult cats under ether anesthesia. 6 had their spinal cord transected through the dorsal atlanto-occipital membrane, 3 were decerebrate precollicularly and 6 were prepared for continued anesthesia by i.v. injections of chloralose (60–80 mg/kg b.wt.). At least 2 h were allowed for recovery after the ether anesthesia before the experiments started. Artificial respiration was instituted only to the preparations made spinal. The condition of the preparations was tested by studying the quadriceps and triceps surae reflex responses to tendon taps or to electrical dorsal root stimulation. Experimental data were collected from preparations in which these tests showed that stretch reflexes or monosynaptic reflexes could be obtained.

In spinal and anesthetized animals a midline skin incision was made, extending from the upper thoracic to the sacral region, and then the fascial layers in the lumbar region and the latissimus dorsi were cut through bilaterally. A laminectomy (L_4 – L_5) was performed. The longissimus and iliocostalis muscles were separated from the iliac bone together with a small segment of the bone which was to be attached to the recording and pulling devices. The animals were fixed in a rigid frame as described in an earlier paper (Carlson 1978 a).

In experiments on decerebrate cats the muscle insertions were initially left intact so that the muscle length could be varied by manually changing the posture of the spine. The position of the head relative to the trunk was not affected by these manipulations. Reflex responses to sustained muscle stretch were also studied, applying procedures similar to those described for the other types of preparations.

The skin was sewn onto the frame to form a pool which was filled with liquid paraffin. The temperature in the pool was kept at 36–38°C by an infrared heating lamp.

Stimulation: In order to stretch the muscles a solenoid (Billman, type RD) was connected in series to an isometric strain gauge transducer (Grass FTO3) which was attached to the isolated segment of the iliac bone by a steel wire (\varnothing 0.6 mm). The solenoid was fed by a Grass S4 stimulator. Usually the amplitudes of the stretches ranged between 0.1 and 0.5 mm, but stretches of larger amplitudes (up to 4 mm) were also applied. The duration of the stretches was up to about 15 ms. The amplitudes and the durations of the stretches used in this study are within the same range as those used by Lloyd (1943) for elicitation of stretch reflex responses. More recent investigations of the spindle afferent activity in response to stretch have shown that primary endings from the cat's soleus muscle are activated by very small stretches (below 60 μ m, Lundberg and Wlansbury 1968) and that also secondary endings may discharge in response to small stretches (Stuart et al. 1970). In view of these results it appears reasonably safe to conclude that the endings of the back muscle spindles have responded to the stretch stimulus used.

For electrical stimulation of dorsal roots, square waves of 0.05–0.1 ms duration were delivered from a Grass S4 stimulator to bipolar silver-silver chloride wire electrodes.

Recording: The arrangement with muscle, transducer and solenoid in series permitted recordings of the time course of the stretches as well as of the subsequent contractions. Electrical reflex responses were

isolated from the muscle in bipolar concentric needle electrodes (DISA[®]). The EMG method was used in it as far as possible as accurate localization of reflexly activated muscle fibers within the muscle belly is not possible. A major factor lowering the use of EMG is the course of the back muscle nerves: since the thin branches are immediately they are difficult to identify and liable to be damaged during the preparation. The results of the EMG for latency measurements will be evaluated in Results. Afferent activity in dorsal roots was recorded by pairs of silver-silver chloride (Ag-AgCl) electrodes.

The signals were fed into Grass P6 amplifiers via cathode followers, displayed on Tektronix dual-beam oscilloscope and recorded by Tinsberg instrumentation recorder.

Results

Characteristics of reflex responses to brief muscle stretch and dorsal root stimulation in spinal and anesthetized preparations

The longissimus and iliocostalis muscles insert onto an intermuscular septum which runs along the middle and lower lumbar region and attaches to the iliac crest. Thus, pulling at a isolated segment of the iliac crest (*cf* Methods) results in a stretch of both muscles. In response to a brief stretch involving both muscles a reflex contraction is evoked as illustrated in Fig. 1 A. The lower record shows the time course of the stretch (initial upward deflection) as well as of the subsequent contraction (*cf* Methods) and the upper record represents the electrical response in the central part of the longissimus at the level of the 5th lumbar vertebra. From the figure the contraction time of reflexly activated motor units can be estimated: the time from the EMG response to peak contractile tension is about 70 ms. In the experiments contraction times of up to 100 ms have been recorded following activation by stretch or dorsal root stimulation (*cf* also Carlson 1978 a).

The amplitude of the EMG response was always largest in the central part of the longissimus along the middle and lower lumbar region as compared with other parts of this muscle and the iliocostalis. Even with larger amplitudes of muscle stretch (*cf* Methods) or when stretch was applied to the fascial layers of the back region in order to involve other portions of the muscles as well, no reflexes could be recorded in other parts than the central region of the longissimus.

The latency of the reflex in the longissimus in response to stretch appears from Fig. 1 B, in which the lower record shows the time course of the muscle stretch and the upper trace the EMG response. The latency of the electrical response from onset of stretch amounts to about 6 ms. The variation of the reflex latency is one and the same preparation was generally below 1 ms but the latency ranged from 4.5 to 7.0 ms in the different experiments. Only in one out of 10 preparations was a latency below 4 ms observed.

In order to determine the central delay of the reflex responses to stretch in the longissimus it was necessary to measure the time periods required for afferent and efferent conduction. By differentiation of various segments it was found that the afferent loop of the reflex arc passes in the L₄ and L₅ dorsal roots. Since it has previously been shown that nerve fibers supplying slowly contracting longissimus muscle fibers run in the L₄ and L₅ ventral roots (Carlson 1978) it seems obvious that the reflex is mediated through these two segments.

The afferent volley recorded from whole dorsal roots or root filaments in response to stretch of the back muscles under study was small in comparison with the volley evoked by



Fig. 1 Reflex contraction of back muscles in response to brief stretch applied to longissimus and iliocostalis muscles. Upper traces. EMG recordings from central region of longissimus at level of 5th lumbar vertebra. Lower traces. mechanical recordings of stretch (upward deflection) and reflex contraction (seen only in A). Time to peak tension in reflex contraction about 70 ms (A). Latency of reflex discharge from onset of stretch about 6 ms (B). Time bar in A 20 ms, in B 5 ms.

a tap applied to the quadriceps tendon. This may be due to the presence of only a small number of spindle afferents from the longissimus and iliocostalis muscles. In Fig. 2 A the upper record shows the afferent response in a filament of the 5th lumbar dorsal root and the lower record the time course of the muscle stretch. A marked increase of the activity is seen about 1–2 ms after the onset of the stretch, which was a typical finding (cf. below).

The efferent conduction time was measured by stimulating cut ventral roots and recording the electrical response within the muscles (cf. Methods). A typical recording of such a response in the central region of the longissimus to stimulation of the 5th lumbar ventral root is illustrated in Fig. 2 B. The time from the stimulus artefact to the first EMG deflection is about 1.4 ms. In the different experiments these values ranged from 1.3 to 1.7 ms. It may be argued that the latency of the EMG response is dependent on the recording site within the muscles: it should be shorter in recordings near the end plate region than from more remote regions. In order to evaluate an error of this type latency measurements were made at high amplification and from different regions in the muscle. Since however the variations thus observed were small the conclusion can be drawn that a reasonably good precision was attained.

Adding the time for the afferent conduction, about 1.0 ms, to that of the efferent conduction, about 1.4 ms, yields a total peripheral conduction time of about 2.4 ms. If the central conduction time for monosynaptic transmission, 1.1 ms according to Lloyd (1943), is added to this value the total reflex time should amount to about 3.5 ms. A latency in this range has occasionally been observed which indicates that monosynaptic reflex pathways do exist (cf. above). As a rule, however, as pointed out above, reflex responses to stretch had longer

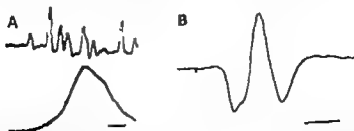


Fig. 2 A afferent volleys recorded from filament of L₅ dorsal root (upper trace) in response to stretch of longissimus (lower trace). Time from onset of stretch to first volley about 1 ms. B, EMG recordings from central region of longissimus in response to supramaximal stimulation of L₅ ventral root. Time from stimulus artefact to first EMG deflection 1.4 ms. Time bars 2 ms.

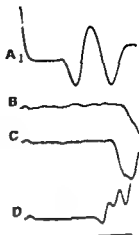


Fig. 1. EMG reflex discharge in central part of longissimus at level of 5th lumbar vertebra in response to dorsal root stimulation. *A*, L_5 dorsal root stimulation. *B-D*, from another experiment. In *B* and *C* stimulation applied to L_1 and L_4 dorsal root respectively. In *D* simultaneous stimulation of these roots. Note decrease in reflex latency when both roots are stimulated. Time bar 2 ms.

latencies, from 4.5 to 7.0 ms, which corresponds to central conduction times in the range from 2 to 5 ms.

The afferent volleys recorded from dorsal roots in response to stretch had a minimum latency of about 1 ms. This excludes the possibility that there should be a long delay between the onset of the stretch and the activation of afferent fibers which would contribute to a long total reflex time. However, it might well be that the first afferent volley in response to stretch is subliminal and that the motoneurons do not discharge until on arrival of subsequent volleys. If this should be the case, there is a possibility that a synchronous afferent volley elicited by electrical stimulation of dorsal roots should give rise to reflexes of considerably shorter latencies than reflexes elicited by a muscle stretch.

A synchronous volley in the 5th lumbar dorsal root may occasionally elicit reflex discharges with a latency of about 2.5 ms (Fig. 3 *A*) but more common is a response of longer latency about 4.0 to 6.0 ms (Fig. 3 *B-D*). The recordings in *B-D* are all from the same experiment and illustrate the EMG response to stimulation of the L_1 root (*B*), the L_4 root (*C*) and to simultaneous stimulation (*D*). The latency of the reflex in *D* is about 1 ms shorter than that in *C* and 2 ms shorter than in *B*. Apparently spatial summation enhances the central reflex transmission. Orthodromic high-frequency stimulation over a brief period of time is known to potentiate the monosynaptic pathway (Lloyd 1949) to hind limb muscles. Such stimulation did not result in any remarkable change of the reflex in the longissimus neck.

To sum up the experiments using adequate as well as those with electrical stimulation indicate that reflex responses recorded from the longissimus have only exceptionally a latency compatible with a monosynaptic reflex transmission. In most experiments a longer latency was recorded which appears to be due to a longer central delay.

Demonstration of tonic stretch reflexes in decerebrate preparations

The central part of the longissimus exhibits prominent tonic stretch reflex. This appears from Fig. 4 in which the upper traces show EMG recordings from that part of the muscle

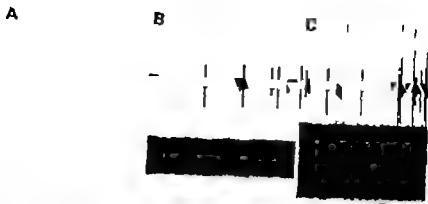


Fig. 4. EMG discharges (upper traces) from central region of longissimus at level of 5th lumbar vertebra on stretch of muscle in steps of 2 mm (lower traces). Length in A shorter than the resting length. Time bar 100 ms.

at the level of the 5th lumbar vertebra and the lower traces represent the relative muscle lengths. At a length shorter than the resting length (A) there is no activity while a recruitment of motor units occurs when the muscle is stretched in steps of 2 mm (B C). No such recruitment was observed in simultaneous recordings from peripheral parts of the longissimus or from various regions of the iliocostalis.

The differentiation of the longissimus muscle into a central slowly contracting portion readily activated by steady stretch, and a peripheral fast contracting part with a high threshold for activation by maintained stretch is of interest from a functional point of view. In a previous investigation of the anatomy and contraction properties of back muscles the longissimus was found to be able to control movements involving the pelvic girdle in relation to the lumbar spine (Carlson 1978 a). The tonic stretch reflex can be utilized to obtain further information about the muscular control of the axial skeleton by studying its variations in the intact muscle when the spine is passively brought into different postures affecting the muscle length. As demonstrated in the previous paper the mobility of each lumbar intervertebral joint is smaller than that of the lumbosacral joint. When the latter joint is passively flexed, resulting in a stretch of the longissimus, a tonic activity in the central region is set up or increased. Conversely when the joint is passively extended the tonic activity invariably decreases or ceases. This is illustrated in Fig. 5 A showing EMG responses obtained during flexion (upper trace) and extension (lower trace) of the lumbosacral joint. In these positions of the joint the peripheral parts of the longissimus as well as the iliocostalis appear to be inactive. A change of the position in the vertical plane in other lumbar joints proved to be less effective in altering the degree of the tonic activity in the central part of the muscle. This portion of the muscle thus seems to be specifically engaged in the tonic control of the lumbosacral joint position or in other terms, in the control of the posture of the lower lumbar spine versus the pelvic girdle. A stretch of the muscle is brought about by flexion in the joint and elicits a reflex contraction opposing the intended movement.

A muscle stretch occurs also on a horizontal bending of the spine, and this elicits a reflex response in the central part of the muscle in the same way as does a vertical bending. The mobility of the lumbar vertebral column in the horizontal plane is small but evidently the



Fig. 1. ENG activity in central longissimus at level of 5th lumbar vertebra when axial skeleton is forced into 4 flexed postures. A, upper record, lumbo-sacral joint flexed; lower record, same joint extended. B, simultaneous recordings from muscles at both sides when lower lumbar spine is bent toward one side in the lumbar plane. Marked activity in contralateral muscle (upper trace), ipsilateral muscle (lower trace) is noted. Time bar 100 ms.

muscle stretch thus induced is large enough to activate the muscle. In horizontal bendings of the spine the longissimus muscles at both sides display a reciprocal activity pattern. When the lower lumbar region is passively bent toward one side a tonic activity is set up in the contralateral muscle, as illustrated in the upper trace of Fig. 5 B, whereas the ipsilateral muscle is inactive (lower trace). This implies that the muscle at one side should be capable to bend the spine toward the same side when the pelvic bone is fixed.

The experiments thus show that the central regions of the longissimus muscles at both sides of the spine act in synergy when vertical flexions of the lower part of the spine are to be counteracted, whereas they act as antagonists in horizontal bendings.

Discussion

The results presented in this study indicate that a selective activation of slowly contracting motor units in the central part of the longissimus muscle occurs in response to brief as well as to sustained stretch. No reflex responses have been obtained from the faster contracting units of the longissimus or from the iliocostalis (contraction times about 30 ms, cf. Carlson 1978 a). This selective reflex activation may be due to several factors but one obvious cause may be the different distribution of muscle spindles, the central part of the longissimus having more spindles than other regions of the two muscles (Carlson 1978 b).

The central region of the longissimus is made up of a high proportion of fibers which are light for myofibrillar ATPase (type I) whereas fibers staining darkly for the ATPase (type II) are predominant in the peripheral longissimus as well as in the whole iliocostalis. A similar uneven distribution of various fiber types is present in some extremity muscles, as e.g. the quadriceps and the tibialis anterior and differences in reflex thresholds have been observed for different regions within these muscles (Denny-Brown 1929, Gordon and Phillips 1933). As demonstrated by Henneman *et al.* (1965) and by Burke (1968), the small low-threshold units in limb muscles have the lowest threshold for reflex activation by stretch. It is evident that the longissimus together with the iliocostalis forms a muscular system consisting of a small, slowly contracting component with a low reflex threshold for stretch and large, fast contracting component which is not recruited by the stretch stimuli used in the present investigation.

In the previous study of the morphology and contraction properties of the back muscle the longissimus and the iliocostalis were found to be involved in the control of the posture of the lumbar spine versus the pelvic girdle (Carlson 1978 a). The effects observed when stretching the longissimus muscle in the decerebrate preparation by forcing the lower spine into different postures suggest that the slowly contracting component of the longissimus may be specifically engaged in the tonic control of the vertical position of the lumbosacral joint. This part of the muscle also exerts a tonic control of horizontal bendings in the low lumbar spine.

It is generally accepted that the reflex response to brief stretch in extremity muscles is set up by monosynaptic excitation from Ia afferents. A monosynaptic pathway from primary spindle afferents does probably also exist for the longissimus since appropriate latencies have been observed experimentally on application of adequate as well as electrical stimulation. A reflex transmission through such a pathway seems however to be exceptional.

As mentioned in the introduction this appears to be the case also as far as the dors neck muscles in the neck region are concerned, as shown by Abrahams, Richmond and Roe (1975). In a pool of 96 motoneurons supplying these muscles only one could be activated by dorsal root stimulation with a central delay shorter than 2 ms at a stimulus strength sufficient for activation of group I and II fibers. 26 neurons were activated after 7-25 ms. Their analysis offers no information about dorsal neck muscle reflexes in response to stretch. Recent experiments (Carlson and Skoglund, unpublished) indicate that these muscles are activated by a brief muscle stretch with a latency in the same range as that usually observed for the longissimus muscle.

In most experiments the shortest central delay of reflexes in the longissimus elicited by stretch as well as by electrical stimulation was long enough to give time for transmission through a polysynaptic pathway. In flexors of the hind limb autogenetic excitation can be set up from secondary endings (cf. Laporte and Bessou 1959; Eccles and Lundberg 1959) and a similar mechanism might be responsible for the reflex activation of the longissimus muscle. Such excitatory reflex effects may via polysynaptic routes, add to a subthreshold monosynaptic Ia excitation and cause a discharge of the motoneurons after a longer delay than that of a monosynaptic reflex. However, an activation of other sense organs by brief stretch is of course plausible, and the possibility that the reflex discharges observed are due to the central action of other receptors than the spindles (cf. Paintal 1960) can therefore not be entirely excluded.

Another mechanism that must be taken into consideration is reflex activation by polysynaptic autogenetic group Ia excitation (for ref. see Homma, 1976). Some evidence for excitatory synaptic actions on hind limb motoneurons by such a mechanism has recently been presented by Taylor *et al.* (1976). Reflex discharges after very long latencies (50-80 ms) following stimulation of hind limb Ia afferents have been demonstrated by Hultborn, Wigström and Wängberg (1975). As far as the central reflex mechanisms of lumbar back muscles are concerned it is not possible to draw any definite conclusions at the present stage. Further experimentation, preferably using intracellular recordings from the back muscle motoneurons, is required.

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mode of work. Thus at submaximal intensities (30–85% VO₂ max) the loss of glycogen occurs mainly in type I fibres, whereas type II fibres are depleted only when the exercise is prolonged (Gollnick *et al.* 1973, Gollnick, Piehl and Saltin 1974). With intense work loads a glycogen depletion is found in both type I and type II fibres, although the depletion pattern varies with the mode of work. Thus a difference between intermittent and continuous exercise has been described, in that during intermittent exercise the glycogen depletion is more pronounced in fibres with the darker NADH-dehydrogenase-stainability *i.e.* those with a higher oxidative capacity (Edgerton *et al.* 1975).

Estimation of glycogen depletion by a histochemical technique is at the best semi-quantitative and detailed quantitative information cannot be obtained as differences in the staining intensity above 80 mmol/kg wet weight are difficult to detect (Piehl 1974). The purpose of the present study was therefore to investigate, in greater detail by quantitative analysis, the glycogen content in type I, II A and II B fibres at rest, as well as the changes of glycogen in these fibres during various types of exercise. A comparison was made of glycogen depletion during intermittent and continuous exercise at high work intensities with continuous exercise at moderate work intensity.

Subjects

Eight healthy volunteers participated in the study. Mean values and ranges were for age 24 (21–41) years, height 178 (174–192) cm, weight 75 (62–87) kg and measured oxygen uptake 3.89 (2.92–4.21) l/min. All subjects were well informed about the procedures to be used before giving their oral consent to participate in the study.

Procedure and Methods

Maximal oxygen uptake during bicycling at 60 rpm for the 13 subjects was determined in a preparatory test. All experiments were performed on a Sarsgaard-Eleonor bicycle ergometer in the sitting position.

In order to study the glycogen depletion in the two main fibre types (I and II), the following experiments were performed. 5 subjects performed intermittent submaximal exercise (15 work 15 rest) for 60 min at the work intensity which demanded the subject maximal oxygen uptake during continuous exercise. At a later test in the laboratory the same 5 subjects performed continuous submaximal exercise for 60 min at approximately half the work intensity used in intermittent exercise. Oxygen uptake was determined after 15, 30 and 45 min duration in all situations. Data on overall substrate utilization from these experiments have been presented previously (Ekel, Hagström and Kjaer 1977).

In order to analyse the glycogen depletion not only in type I and II fibres but also in the subgroups of type II fibres, some additional similar experiments were performed. 3 subjects performed intermittent submaximal exercise for 60 min and 2 subjects continuous submaximal exercise for 60 min. Finally 3 subjects performed continuous submaximal exercise at a similar work intensity to that in the experiments with intermittent exercise, thus led to exhaustion after 4–6 min.

Muscle biopsies from the vastus lateralis were obtained at rest and at the end of all the experiments and in some subjects also after 5 and 30 min duration of the exercise.

Analysis. Expired air was collected in Douglas bags. The volume of the air was measured in a spirometer and was analysed for the contents of O₂ and CO₂ with the Haldane technique. Muscle samples were obtained with percutaneous biopsy technique (Bergström 1962) and were frozen immediately in liquid nitrogen and were stored at -80°C until analysed. After freeze-drying of the muscle samples, individual fibre fragments were dissected out (100–200 fibres from each sample). In order to identify the fibre fragments, 2–3 small pieces were cut off each one and stained for myofibrillar ATPase after preincubation at pH 10.3, 4.6 or 4.3 (Ekel *et al.* 1975). The remaining part of the fibre was weighed on a quartz fibre balance, and its glycogen hydrolysed in 1 M HCl at 100°C for 2 h. The samples were then cooled and aliquots were taken for subsequent analyses of glucose residues (Ekel and Himmelman 1975).

Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exercise

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Abstract

ESSÉN B. Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exercise. *Acta physiol. scand.* 1978. 103. 446-455.

Muscle biopsies from the vastus lateralis were obtained from thirteen healthy subjects at rest and at rest during continuous and intermittent exercise. After freeze-drying of the muscle sample, fragments of single fibres were dissected out and stained for myofibrillar ATPase with preincubation at pH 10.3, 4.6 or 4.3 to identify type I, II A and II B fibres respectively. The remaining part of each fragment was used for quantitative glycogen analyses. At rest, mean glycogen content was significantly higher in type II fibres (40 mmol/kg dry weight) than in type I (34 mmol/kg dry weight). After 60 min continuous exercise at moderate work load a more pronounced glycogen depletion had occurred in type I (27 mmol/kg dry weight) than in type II (A + B) fibres (11 mmol/kg dry weight). With 60 min intense intermittent exercise a significant, but similar depletion occurred in both type I (23 mmol/kg dry weight) and type II (A + B) fibres (20 mmol/kg dry weight). With continuous intense exercise to exhaustion (4-6 min), glycogen depletion was more marked in type II (A + B) (18 mmol/kg dry weight) than in type I fibres (7 mmol/kg dry weight). These data imply that the glycogen depletion pattern in muscle fibres is determined mainly by the work intensity but the lower glycogen depletion per unit time in intermittent compared with continuous intense exercise indicates that the mode and duration of exercise is important, too.

Glycogen utilization in human skeletal muscle varies with the intensity, duration and mode of exercise, which to some extent seems to be due to a pattern of selective recruitment of muscle fibres with different metabolic characteristics. Based on stainings for myofibrillar ATPase activity the fibres of human skeletal muscle can be classified into two main groups, type I and II (Engel 1962). Type I fibres usually have the higher oxidative capacity, type II fibres the higher glycolytic capacity (Dubowitz and Brooke 1973). Type II fibres can be subclassified into type II A and II B (Brooke and Kaiser 1970), the former apparently having the higher oxidative capacity and the latter the higher glycolytic capacity (Essén *et al.* 1975). In spite of these differences in glycolytic potential, no systematic quantitative difference in basal glycogen content has been established between the different fibre types in man.

During exercise, glycogen seems to disappear at different rates in the different fibre types based on histochemical data and these differences are dependent on both load and

TABLE II
Oligomers content (mole %) in oligomer
samples (160 wt%).

Intermittent intense exercise (15 weeks, 12 days)														
Subject	Fibre type distribution	5 min						30 min		60 min				
		Rest		5 min		30 min		60 min		30 min		60 min		
		I	II A	II B	I	II A	II B	I	II A	I	II B	I	II A	II B
6	I	378 ±134 (25)	407 ±119 (79)	467 ±79 ^b (17)	346 ±90 ^a (32)	326 ±94 ^a (40)	311 ±93 ^a (19)	219 ±112 ^a (59)	234 ±103 ^a (36)	215 ±98 ^a (12)	117 ±103 ^a (79)	183 ±137 ^a (45)	226 ±143 ^a (26)	
	II	21	21	467 ±79 ^b (17)	346 ±90 ^a (32)	326 ±94 ^a (40)	311 ±93 ^a (19)	219 ±112 ^a (59)	234 ±103 ^a (36)	215 ±98 ^a (12)	117 ±103 ^a (79)	183 ±137 ^a (45)	226 ±143 ^a (26)	
7	I	388 ±106 (39)	386 ±123 (20)	396 (8)	334 ±103 ^a (26)	376 ±88 (36)	375 ±145 (16)	182 ±57 ^a (60)	287 ±63 ^a (46)	253 ±64 ^{a,b} (38)	92 ±71 ^a (31)	169 ±139 ^a (39)	119 ±72 ^a (16)	
	II	16	16	396 (8)	334 ±103 ^a (26)	376 ±88 (36)	375 ±145 (16)	182 ±57 ^a (60)	287 ±63 ^a (46)	253 ±64 ^{a,b} (38)	92 ±71 ^a (31)	169 ±139 ^a (39)	119 ±72 ^a (16)	
8	I	348 ±99 (32)	348 ±99 (30)	396 ±70 ^b (20)	—	—	—	—	—	—	141 ±75 ^a (86)	255 ±181 ^{a,b} (75)	304 ±176 ^b (47)	
	II	15	15	396 ±70 ^b (20)	—	—	—	—	—	—	141 ±75 ^a (86)	255 ±181 ^{a,b} (75)	304 ±176 ^b (47)	
9	I	413 ±76 (25)	444 ±86 (24)	483 ±34 (10)	346 ±58 ^a (34)	478 ±32 ^a (26)	467 ±82 ^b (11)	—	—	—	69 ±45 ^a (25)	435 ±127 ^a (39)	289 ±141 ^{a,b} (21)	
	II	8	8	483 ±34 (10)	346 ±58 ^a (34)	478 ±32 ^a (26)	467 ±82 ^b (11)	—	—	—	69 ±45 ^a (25)	435 ±127 ^a (39)	289 ±141 ^{a,b} (21)	
10	I	513 ±70 (12)	599 ±87 (25)	640 ±73 ^b (17)	—	—	—	—	—	—	84 ±57 ^a (33)	345 ±193 ^{a,b} (33)	446 ±121 ^{a,b} (16)	
	II	12	12	640 ±73 ^b (17)	—	—	—	—	—	—	84 ±57 ^a (33)	345 ±193 ^{a,b} (33)	446 ±121 ^{a,b} (16)	

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Ullrich mean values in type II A cases	Ullrich mean values in type II B cases	Ullrich mean values in type II C cases	Ullrich mean values in type II D cases
0.005	0.005	0.005	0.005

Highest mean value in type II B than type I fibers ($p = 0.007$)

the other nuclei values in type II is than type

^a Some mean values are less than standard error.

In order to establish the percentage of each fibre type in each subject, part of a muscle sample was prepared for histochemical analyses and serial cross sections were stained for myofibrillar ATPase after acid and alkaline preincubation (Brooke *et al.* 1970).

Statistics. The significance of differences between categories was determined according to Student's *t*-test and the rank sum test (Dixon and Massey 1957).

Results

Work intensity

In intermittent exercise, mean work intensity and oxygen uptake were 299 (range 270–343) W and 2.21 (1.93–2.81) l/min, respectively. In continuous submaximal exercise they were 157 (138–180) W and 2.31 (1.93–2.84) l/min, and in continuous maximal exercise 266 (220–300) W and 3.56 (2.92–3.94) l/min. Mean oxygen uptake values corresponded to 50–60% of the subject's maximal oxygen uptake ($\dot{V}O_{\max}$) in both intermittent intense and continuous submaximal exercise, whereas the corresponding value during continuous maximal exercise was 100% $\dot{V}O_{\max}$.

Glycogen content at rest (Table I–III)

At rest a large variation in glycogen content was found in all subjects within type I, II A and II B fibres respectively and the values were distributed symmetrically around the mean (Fig. 1 and 2). The mean concentration was higher in type II than in type I fibres in all but one subject. The mean value and range for type I fibres, 344 (223–513) mmol/kg dry weight, was significantly lower ($p < 0.001$) than in type II fibres, 402 (271–610) mmol/kg dry weight. No significant difference was found between the mean glycogen content of type II A and type II B fibres, 430 (335–589) and 454 (350–640) mmol/kg dry weight.

TABLE I Glycogen content (mean \pm S.D.) in type I and II fibres at rest and after 60 min intermittent (15 s work–15 s rest) intense exercise (299 watt) and continuous submaximal exercise (157 watt).

Subject	Fibre type distribution %			Intermittent intense exercise (15 s work–15 s rest)				Continuous submaximal exercise			
				Rest		60 min		Rest		60 min	
	I	II A	II B	I	II	I	II	I	II	I	II
1	63	33	4	292 \pm 75 (28)	377 \pm 79 ^a (37)	120 \pm 101 ^b (29)	252 \pm 123 ^b (52)	288 \pm 70 (47)	408 \pm 91 (44)	95 \pm 130 ^b (28)	362 \pm 199 (28)
2	57	32	11	413 \pm 121 (37)	441 \pm 102 (60)	89 \pm 72 ^b (33)	187 \pm 105 ^b (69)	303 \pm 64 (57)	179 \pm 89 ^a (41)	36 \pm 60 ^b (68)	251 \pm 102 ^b (34)
3	49	45	6	283 \pm 64 (29)	271 \pm 44 (60)	46 \pm 63 ^b (33)	39 \pm 56 ^b (73)	400 \pm 57 (74)	407 \pm 70 (62)	94 \pm 99 ^b (91)	189 \pm 111 ^b (74)
4	52	38	10	250 \pm 53 (41)	344 \pm 78 ^a (58)	197 \pm 100 ^b (110)	109 \pm 91 ^b (107)	360 \pm 64 (45)	381 \pm 73 (52)	131 \pm 94 ^b (73)	388 \pm 142 (65)
5	54	29	13	279 \pm 81 (20)	302 \pm 56 (75)	148 \pm 80 ^b (76)	96 \pm 81 ^b (109)	355 \pm 79 (39)	427 \pm 105 ^a (43)	184 \pm 164 ^b (46)	320 \pm 117 ^a (64)

Higher mean value in type II than type I fibres ($p < 0.05$).

^a Lower mean value than at rest ($p < 0.05$).

Numbers within parentheses denote the number of fibres analysed for glycogen.

TABLE III Glycogen content (mean \pm S.D.) in type I, II A and II B fibres at rest and at end of (4-5 min) continuous intense exercise to exhaustion (267 watt).

Subject	Fibre type distribution %			Continuous intense exercise to exhaustion					
				Rest			Exercise (4-5 min)		
	I	II A	II B	I	II A	II B	I	II A	II B
11	35	50	15	223 \pm 131 (44)	335 \pm 142 ^a (61)	350 \pm 121 ^b (24)	137 \pm 45 ^d (24)	46 \pm 48 ^{a,d} (36)	207 \pm 54 ^{a,d} (24)
12	61	30	9	409 \pm 79 (23)	470 \pm 106 (18)	515 \pm 76 ^b (9)	359 \pm 70 ^d (30)	396 \pm 82 ^{a,d} (30)	441 \pm 60 ^{a,d} (14)
13	73	25	2	284 \pm 71 (41)	419 \pm 60 ^b (37)	383 \pm 43 ^b (14)	199 \pm 70 ^d (99)	239 \pm 88 ^{a,d} (47)	40 \pm 49 ^{b,d} (7)

^a Higher mean value in type II A than type I fibres ($p < 0.05$).

^b Higher mean value in type II B than type I fibres ($p < 0.05$).

^c Higher mean value in type II B than type II A fibres ($p < 0.05$).

^d Lower mean value than at rest ($p < 0.05$).

Numbers within parentheses denote the number of fibres analysed for glycogen.

Glycogen depletion during continuous submaximal exercise (Table I, II)

At the end of the continuous submaximal exercise a mean decrease of 277 (171-344) mmol/kg dry weight ($p < 0.001$) was found in type I fibres and 113 (-7-225) mmol/kg dry weight ($p < 0.05$) in type II fibres (Fig. 1). The depletion was more pronounced in type I than in type II fibres ($p < 0.01$). The pattern for glycogen content in the different fibre types, as shown in Fig. 1 indicates that most of the type I fibres had lost glycogen with this mode of exercise but only some of the type II fibres.

The two subjects in whom glycogen was analyzed separately in type II A and II B fibres showed great variations in their fibre type distribution as well as in their glycogen depletion pattern (Table II). Thus the subject with 64% type I fibres showed a significant decrease of glycogen in these fibres as well as in the few type II B fibres, while no decrease was seen in type II A fibres after 60 min. The other subject, who had only 18% type I fibres, showed a significant decrease in glycogen in all 3 types of fibres after 60 min.

Glycogen depletion during intermittent intense exercise (Tables I, II)

After 60 min intermittent exercise the mean decrease was in type I fibres 213 (53-344) mmol/kg dry weight ($p < 0.001$) and in type II fibres 203 (125-254) mmol/kg dry weight ($p < 0.001$). In the 3 subjects where glycogen was analysed in the subgroups of type II fibres, a decrease had occurred at the end of exercise in both type II A and II B fibres within each subject ($p < 0.05$) (Fig. 2). The mean decrease was in type II A 185 (113-224) mmol/kg dry weight and in type II B 204 (94-277) mmol/kg dry weight.

Glycogen depletion during continuous intense exercise to exhaustion (Table III)

A significant glycogen decrease was found in type I, II A and II B fibres in each of the 3 subjects who performed continuous exercise to exhaustion. The mean decreases in the 3

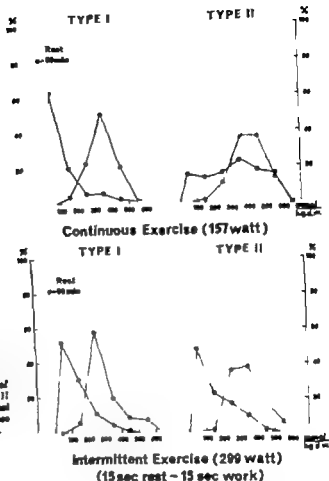


Fig. 1. The distribution pattern of glycogen content in type I and II fibres with continuous submaximal exercise and intermittent submaximal exercise (mean from 5 subjects).

types were 74 (50–86), 114 (74–180) and 120 (74–143) mmol/kg dry weight respectively. In all 3 subjects the glycogen decrease was greater in type II than in type I fibres. The distribution pattern of glycogen content in the different fibre types indicates that within each fibre type most fibres had lost some glycogen with intense exercise (Fig. 2).

The glycogen depletion in type II A fibres, was 2 times greater for the subject with the lowest percentage of these fibres (25%) than in the subjects with the highest (50%).

Discussion

Quantitative analysis in single fibre fragments showed at rest a slightly greater mean glycogen content in type II than in type I fibres. With continuous prolonged exercise for 60 min at moderate work intensity glycogen depletion was more pronounced in type I than in type II fibres. At high work intensity performed either as continuous or as intermittent exercise, both type I and type II fibres were glycogen depleted. The intermittent intense

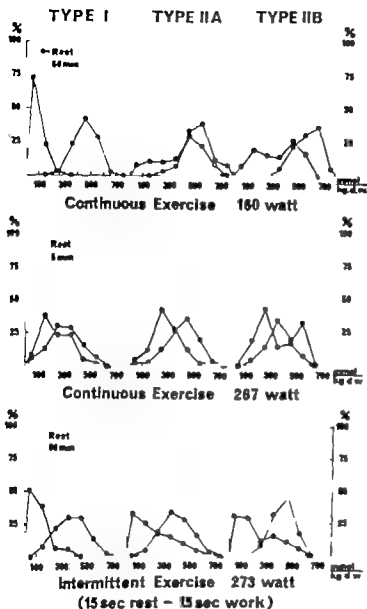


Fig. 4. The distribution pattern of glycogen content in type I, IIA and IIB fibres with continuous submaximal exercise (mean of subjects), continuous intense exercise (mean of 3 subjects) and intermittent intense exercise (mean of 3 subjects).

exercise, however, gave a smaller depletion per unit time than continuous intense exercise. The data thus imply that the glycogen depletion pattern of muscle fibres during bicycle exercise is determined mainly by the work intensity but that the mode and duration of exercise is evidently of importance, too.

Glycogen content at rest

The slightly greater mean resting glycogen content in type II than type I fibres seems to indicate that type II fibres normally have the greater capacity for glycogen storage. However, it is worth emphasizing that the ranges of glycogen content for both the type I and the type II fibre population, are wide and overlapping. When evaluating the initial level of glycogen in different fibre types it must also be remembered that glycogen depletion

already occurs preferentially in the type I fibres at the work intensities associated with daily routine activities. In this study the subjects were all studied in the morning and no intense physical exercise had been performed before the expt. However even physical activity and diet the day before the expt. might be of significance, as it is known that glycogen repletion can be a fairly slow process (Piehl 1974).

Glycogen depletion during continuous submaximal exercise

The marked glycogen depletion in type I fibres and the slight depletion in type II fibres in most subjects after 60 min submaximal exercise is in good agreement with earlier findings using histochemical techniques (Gollnick *et al.* 1973 Gollnick *et al.* 1974). The distribution pattern at rest and at the end of exercise for fibres with different glycogen contents indicates that all of the type I fibres have been involved during submaximal continuous exercise, but only some of the type II fibres. Glycogen depletion in the subgroups of type II fibres has previously been studied, only at higher work intensities ($\sim 85\%$ VO_2 max), using histochemical analysis, which has shown that type II A fibres are more depleted than type II B fibres (Andersen and Sjogaard 1975). The present study shows that in addition to a marked depletion of type I fibres, both type II A and type II B fibres to some extent can be glycogen depleted even at lower work intensities (~ 50 – 60% VO_2 max). The magnitude of this depletion seems to be dependent on the subject's percentage distribution of fibre types, as discussed below.

Glycogen depletion during continuous intense exercise

With maximal exercise ($\sim 100\%$ VO_2 max) glycogen depletion occurred in all three fibre types, suggesting that all these fibres had been activated during some phase of the work. The depletion was more marked in type II than type I fibres. One factor which might contribute to this difference is that type II fibres have a greater glycolytic capacity than type I fibres. Histochemical analyses have also shown a glycogen depletion in both type II and type I fibres, more marked in type II, although these studies were performed at work intensities of 120–140% VO_2 max (Gollnick *et al.* 1973 1974).

Glycogen depletion during intermittent intense exercise

The overall metabolic response in intermittent intense exercise is found to be more similar to continuous exercise of lower intensity than to continuous exercise of the same high work intensity (Åstrand *et al.* 1960, Enén *et al.* 1977). The present study indicates that during intermittent intense exercise, type I II A and II B fibres show a glycogen depletion pattern which is more comparable to that of continuous maximal exercise than continuous submaximal exercise (Fig. 1 2). However with intermittent exercise, glycogen depletion per unit time was lower than with continuous maximal work in both type I and type II fibres and usually of a similar magnitude. These findings seem to indicate that the glycogen depletion pattern is determined mainly by the work intensity although the rate of glycogen depletion is influenced by the mode of exercise, in both fibre types.

The lower glycogen depletion in intermittent exercise may then be explained by a relative increase in lipid contribution to oxidative metabolism, which is suggested to be due to the

increased levels of ATP, CP and citrate at the end of each rest period, thus suppressing glycolysis in the early phase of the subsequent work period (Essén *et al.* 1977). There is also a possible higher oxygen availability due to reloading of myoglobin stores in the resting periods, and subsequently a greater aerobic energy output which gives a higher ATP production per glucose unit as compared to lactate formation.

Glycogen depletion and fibre type distribution in muscle

Subjects with low percentage type I fibres showed different glycogen depletion pattern than those with low percentage type II fibres. Thus with continuous submaximal exercise the subject with a low percentage of type I fibres showed a greater depletion in type II fibres than the subject with a greater percentage of type I fibres, probably because the tension development exceeded the capability of type I fibres already at low work load. With continuous intense exercise the subject with a low percentage of type II fibres showed a greater glycogen depletion in these fibres than the subject with twice as many type II fibres, which indicates that each one of these fibres is involved more often at higher work intensities. The involvement of type II fibres is probably due to the rapid tension development at the start of each pedal thrust and the fact that peak tension amounts to a significant fraction of maximal voluntary contraction, which cannot be achieved by type I fibres alone, as has been shown for submaximal continuous exercise (85% VO max) (Andersen *et al.* 1975).

Glycogen depletion pattern and fibre recruitment

Glycogen depletion indicates that the fibres have been active during some phase of a work bout, contributing to the tension development. However, no exact information can be obtained about the extent of fibre activation as several other substrates than glycogen are available in the muscle cell, such as glucose, fatty acids, and triglycerides; these are probably used at different rates depending on the work intensity, duration of exercise and availability of substrates. This contribution from other substrates may differ between fibre types. As stated above, intermittent intense exercise is a good example of situations where these substrates are of great importance, thus contributing to a glycogen saving effect. Other circumstances which limit the use of glycogen depletion data as an indication of fibre recruitment are that glycogen with complete combustion gives a greater energy release than with lactate formation, that glycolysis might occur without tension output, and that glycogen resynthesis may occur in the muscle cell. However, with all these limitations in mind, glycogen depletion over a substantial period indicates that the fibres have contributed to the total tension output and can thus be taken as an index for fibre recruitment.

A preferential recruitment of type I fibres would thus occur at low work intensity in contrast to recruitment of both type I and type II fibres at higher work intensities. This statement is consistent with the innervation pattern found in the cat, where type I fibres are innervated by small motor neurons with low activation thresholds, whereas motor units with larger motor neurons and high thresholds innervate type II fibres (Henneman and Olson 1965).

During intermittent intense exercise, type II fibres involvement may be due not only to

the intense activity but also to the repeated phases that occur of increased load to accidents from stand still to 60 rpm with each work period. It has been shown in voluntary contraction of the toe extensor muscle, with rapid acceleration phases, that motor units with type II fibres are preferentially recruited before those with type I fibres (Grimby and Hansson 1977).

In conclusion, glycogen depletion occurs mainly in type I fibres with submaximal continuous exercise and in all three types, I, II A and II B, with intense exercise, performed intermittently or continuously. The glycogen saving effect in intermittent exercise is then not dependent on a specific fibre activation pattern.

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Contralateral influence on recruitment of curarized muscle fibres during maximal voluntary extension of the legs

By

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Abstract

SECHER, N. H. & RORSGAARD, S. & SECHER, O. Contralateral influence on recruitment of curarized muscle fibres during maximal voluntary extension of the legs. *Acta physiol. scand.* 1978. 103. 456-462.

The force of maximal voluntary contraction (MVC) was compared during extension of one leg alone and during simultaneous extension of both legs. In 6 subjects MVC of two leg extension was 75 ± 3.6 (S.E.) of the sum of the single one leg MVC. This may indicate a reduced muscle fibre involvement during two leg extension as compared with one leg extension. We investigated the possibility that either red (type I) or white (type II) muscle fibre recruitment was restricted during two leg extension. The neuromuscular transmission in the two types of fibres was partially blocked with d-tubocurarine (dtc) or decamethonium (C_{10}) for either type I or II respectively. In 3 subjects receiving dtc the ratio between the reduced two leg and one leg extension forces ($75 \pm 2.3\%$) did not change. During administration of C_{10} , however, this ratio decreased by $16 \pm 3.1\%$. Partial blocking with dtc caused a two component curve during tension development with a first maximum at 0.22 s and another maximum ± 1.03 s of 3 s attempt to reach MVC. During partial blocking with C_{10} the first maximum disappeared indicating human fast twitch fibres to be blocked by C_{10} . Thus, partial blocking with C_{10} results in muscle contractions where a relatively large amount of type I muscle fibres are contributing to the force developed. The experiment suggests that type I fibre recruitment is restricted during severe static exercise in normal muscles. Furthermore, it demonstrates that fibre recruitment can vary in the same muscle function performed with one leg or two legs.

Key words: d-tubocurarine; decamethonium; rate of rise of tension; fast and slow muscle fibres; motor unit recruitment; motor skill.

The force of maximal voluntary contractions (MCV) is determined by the cross sectional area of the muscles and/or the ability of the nervous system for simultaneous tetanic recruitment of all synergistic motor units. Merton (1954) found the same force in the adductor pollicis muscle during MVC and electrical stimulation of the ulnar nerve. However, in similar experiments Ikal *et al.* (1967) saw a 30% increase in force during electrical stimulation. A study of Ikal and Steinhaus (1961) showed MVC to be influenced by a variety of external stimuli. In untrained subjects the verbal suggestion that strength was increased also increased MVC, but had no effect in a trained subject. Comparison of one and two leg MVC shows a lower two leg MVC than the sum of the two one leg MVC (Asmussen *et al.* 1959; Asmussen and Heeboll Nielsen 1961; Secher *et al.* 1976). This is not the case in subjects (oarsmen) who perform repetitive two leg extension during training.

(Secher 1975). These studies suggest that MVC only reflects full muscle strength in well trained subjects.

The present study was undertaken to investigate the possibility of a restricted red (type I) or late (type II) muscle fibre recruitment during simultaneous extension of the legs in subjects. Its low two to one leg MVC ratio. Partial neuromuscular block can be obtained for mainly type I or mainly type II muscle fibres. D-tubocurarine (dtc) is known to have a preference for neuromuscular transmission in red muscles while primarily white muscles are blocked by decamethonium (C_{10}) as shown in several animal species by Paton and Zaiman (1947). Subsequently Johansen *et al* (1964) and Jørgensen *et al* (1966) found a similar effect of dtc and C_{10} in human forearm and neck muscles. Thus, one and two leg MVC were compared during partial neuromuscular block with dtc and C_{10} . A preliminary note of the results has appeared (Secher *et al* 1976).

Material and Methods

Six subjects (23–24 years), 3 of whom had extensive laboratory experience as subjects, participated in the study after their informed consent was obtained. Height and weight (mean \pm S.E.) were 183 ± 4 cm and 80 ± 11 kg, respectively. The muscle groups tested were the leg extensors.

On the first day the force of MVC was recorded during 3 or 4 successive trials 30 to 60 s apart. A value was accepted as maximum when the following trial showed a decrease in force. Before and during the test the subjects were encouraged by the examiner to perform maximally. MVC during leg extension was carried out on strain gauge dynamometer actometers to Astrand *et al* (1959). During this test the subject is seated on a adjustable seat stretched in a horizontal iron frame. The feet are placed on a steel bar equipped with plates at the center. The bar is mounted in bearings on the frame. The angle of the ankle joints is kept 90° and the knee joint at 90° . Tests were performed as one and two leg MVC.

The strain gauges were connected to a Pickett oscillograph (581 DMK) and output read on an Elema-Schneider recorder at a speed of 2.5 cm s^{-1} .

On the second day force development during MVC was first measured as described above and d-tubocurarine chloride (Abbott) 0.1 mg kg^{-1} (3 subjects) or decamethonium bromide (Borroughs Wellcome) 1.0 to 1.5 mg kg^{-1} (3 subjects) administered through catheter in the left forearm. A similar procedure was followed on the third experimental day on which occurred the alternative drug was used. The experimental days were chosen more than 1 week apart in order to avoid drug accumulation or withdrawal. During the action of the drugs recordings were made with 15 muscle alternating between the right, left or both legs. An Ambre-P. recorder apparatus was kept ready for all experiments, but this was not used in most of the experiments as need for it to be used.

Ordinary statistical methods were used to calculate means and standard error of the means (S.E.). Deviations from the normal distribution was calculated and non-individual differences were evaluated by Student's paired *t*-test (two-tailed).

Results

Maximum force during MVC for the right and the left leg was 150 ± 22 and 130 ± 17 kp respectively ($P < 0.6$). For both legs it was 210 ± 23 kp or 75 ± 3.6 percent of the sum of the two one leg forces.

At the time of maximal action of the drugs the subjects had double vision and difficulty in maintaining head position, but all were ventilating adequately with no clinical sign of respiratory depression. Typical force recordings for a single subject are shown in Fig. 1. The maximum curves obtained are without dtc or C_{10} , respectively. Lower recordings were obtained during the action of the two drugs. Thus, the lowest recordings represented MVC during maximal action of the drugs. All curves were superimposed for direct comparison. At onset of the contraction a rapid and short lasting (easily fatiguable) component

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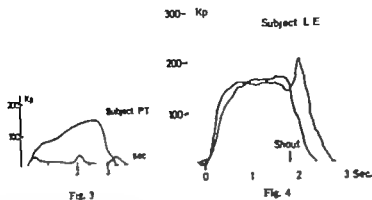


Fig. 3

Fig. 4

Fig. 3 Single recordings of force in uncontracted state and during administration of d-tubocurarine. Note that the fast component appears 3 times during an MVC recording.

Fig. 4 Force development during MVC composed with and without shouting.

Force maximum (control value 1.5 ± 0.18 s) tended to be lower during the partial neuromuscular blockings (for dTC expt. 1.3 ± 0.12 s and for C₅₄ expt. 1.13 ± 0.08 s). The late component of the recordings appeared at a similar time after onset of the contraction during dTC and C₅₄ expts. The early component had vanished at the time when the force of the non-blocked muscle reached the maximum of the late component.

The distributions of the times to the two force maxima for all recordings are shown in Fig. 2. Both the frequency distributions for the early and the late components were skewed to the right indicating an overrepresentation of slow contractions. Medians were (with confidence limits) 0.22 (0.13-0.50) and 1.03 (0.64-2.7) s. Note that the early component only appeared in 411 of 807 recordings.

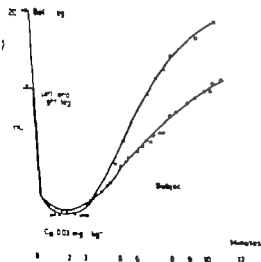


Fig. 5 Force maximum read from MVC recordings made before and after single injection of decamethonium.

Subject ME

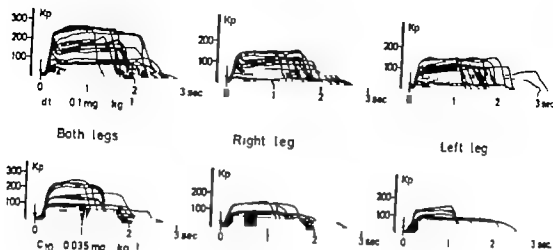


Fig. 1 Force recorded during MVC for one subject during extension right, left and both legs. *Upper tracing* d-tubocurarine expt. *Lower tracing* decamethonium expt. The curves for the expts. are superimposed.

appeared during blocking with dtc. This fast component was less frequent during the action of C_{10} . It was present in $39 \pm 5.9\%$ of the unblocked recordings and more often ($82 \pm 6.8\%$) in the dtc recordings than in the C_{10} recordings ($24 \pm 5.5\%$) ($P < 0.005$). The time to the early force maximum (control value 0.35 ± 0.06 s) was not significantly affected by administration of C_{10} (0.24 ± 0.03 s) or dtc (0.29 ± 0.03 s). The time to the late

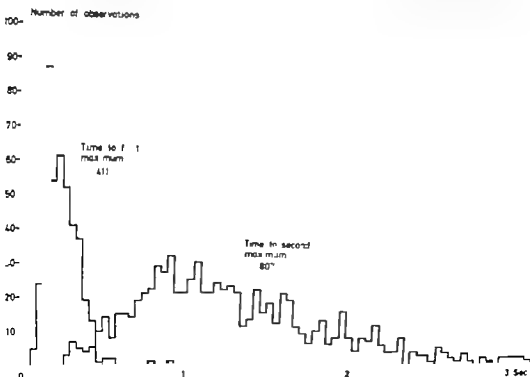


Fig. 2. Frequency distribution of time to maximum for first and second component of force development.

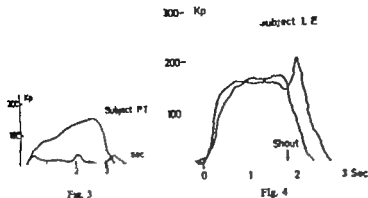


Fig. 3

Fig. 4

Fig. 3 Single recordings of force in vocerized state and during administration of *d*-tubocurarine. Note that the last component appears 3 times during an MVC recording.

Fig. 4 Force development during MVC composed with and without shouting.

force maximum (control value 1.5 ± 0.18 s) tended to be lower during the partial neuromuscular blockings (for disc expt. 1.3 ± 0.12 s and for C_{10} expt. 1.13 ± 0.08 s). The late component of the recordings appeared at a similar time after onset of the contraction for disc and C_{10} expts. The early component had vanished at the time when the force of the non-blocked muscle reached the maximum of the late component.

The distributions of the times to the two force maxima for all recordings are shown in Fig. 2. Both the frequency distributions for the early and the late components were skewed to the right indicating an overrepresentation of slow contractions. Medians were (with confidence limits) 0.22 (0.13–0.50) and 1.03 (0.64–2.7) s. Note that the early component only appeared in 411 of 807 recordings.

22 = Both ex

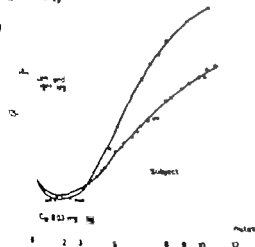


Fig. 5. Force maximum read from MVC recordings made before and after single injection of decamethonium.

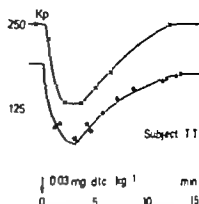


Fig. 6. Force maxima read from MVC recordings made before and after a single i.v. injection of d-tubocurarine. Signs as in Fig. 5.

A fast component reappeared during the same contraction during renewed effort in the subject, but force could not be maintained on this level and a marked fade in contraction was seen in each instance (see dtc recording of Fig. 3). Late appearance of the fast component could also be elicited by exceptional stimulation such as a shout—cf. the recording in the unblocked state in Fig. 4.

The variation in maximum force reached at repeated contractions during drug administration is illustrated in Fig. 5 and 6 for C and dtc, respectively. In the 5 experienced subjects similar smooth curves could be drawn using approximately all force determination as estimates of MVC. But in the inexperienced subject this was not possible, and his data were therefore not used in this part of the study. Occasionally recovery MVC exceeded MVC recorded before drug administration (Fig. 5, one leg values).

Ratios between two and one leg extension forces were similar before and after recovery from drug administration (Table I). The average was used as control value. These ratios were reproducible from day to day. However, two leg maximum force was relatively more reduced during administration of C than one leg maximum force; dtc reduced one and two leg maximum forces to approximately the same extent. Two examples with particularly large falls in force development are shown in Fig. 5 and 6. During maximum effect of C

TABLE I. Ratios between MVC during extension of both legs and MVC of right plus left leg.

		no.	dtc	C ₁₀	Difference	± S.E.	t value
Before medication	mean	5	77	81	-4	1.4	2.66
	± S.E.		4.9	3.6			
After medication	mean	5	74	80	-6	5.2	1.12
	± S.E.		2.2	4.5			
Difference		5	3	1			
	± S.E.		2.1	4.0			
t value			1.41	0.25			
Average of before and after values	mean	5	75	80	-5	3.3	1.43
	± S.E.		2.3	3.5			
During maximal action of the drugs	mean	5	77	64	13	2.6	5.1
	± S.E.		3.8	4.0			
Difference	mean	5	—	16			
	± S.E.		5.7	3.1			
t value			0.37	5.19			

two leg force was equal to one leg force (Fig. 5). During the maximum action of d-tc the two leg to one leg force ratio tended to be higher than that observed in the unblocked state (cf. Fig. 6).

Discussion

Contraction pattern

The times to maximum force observed here for the fast and slow components of force development are in the same order of magnitude as that noted during tetani of fast and slow motor units (Sigg 1964, Close 1967). In the present study the distributions of the times to maximum force were skewed towards the right indicating an overrepresentation of slow elements within both components. If the subjects used an effort less than maximal at onset of contraction, as the uncensored MVC recording of Fig. 3, this will tend to delay the time to maximum force and thereby obscure the two component pattern. In general, the force pattern recorded by us during blocking supports the concept, that a fast and slow type of human muscle fibres work together during voluntary contraction. It also confirms earlier findings of the specificity of a blocking by d-tc and C_{12} of human type I and II muscle fibres, respectively (Molbech and Johansen 1969, Bonde-Petersen *et al.* 1975).

The fast component initiates the contractions recorded. The data of Grimby and Benzerit (1968) suggested a change in the recruitment pattern in human skeletal muscle only during contraction of high intensity (i.e. high velocity). The present data suggest that a gradual change in muscle fibre recruitment takes place during intensive muscular contractions because fatigue in the fast contracting fibres appears rapidly. However the MVC recordings during C_{12} blocking show that slow fibres also can be active at onset of contraction. The fast contracting element only reappears with renewed effort. This may explain the increase in the recorded strength after a shot or a shout as described by Ikai and Prosser (1961).

Thorsen *et al.* (1976) saw a significant correlation between force and type II muscle fibre area only at high contraction velocities. The present recordings support the feeling that the fast contracting fibres are of importance for the rate of rise of tension rather than for the absolute force reached.

Muscle fibre recruitment

The present data (Table I, Fig. 5 & 6) indicate a restricted type I muscle fibre recruitment during simultaneous two leg extension.

Asmussen *et al.* (1959), Asmussen and Heebull-Nielsen (1961) and Secher *et al.* (1976) showed, as confirmed in the present study two leg extension force to be less than the summated one leg extension force except in subjects specially trained for two leg extension (e.g. rowers, Secher 1975). These studies indicate that muscular performance during complex motor activity can be improved by training specifically for one or two leg achievements.

According to Henneman (1957) and Henneman and Olson (1965) it would be expected that type I muscle fibres contained in small motor units with a low activation threshold are active whenever type II fibres are recruited. The present results indicate a restricted type I muscle fibre recruitment during two-legged MVC. However the work of Henneman

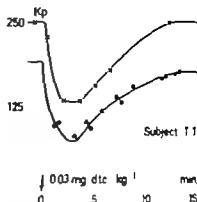


Fig. 6. Force maxima read from MVC recordings made before and after a single i.v. injection of d-tubocurarine. Signs as in Fig. 5.

A fast component reappeared during the same contraction during renewed effort by the subject, but force could not be maintained on this level and a marked fade in contraction was seen in each instance (see dte recording of Fig. 3). Late appearance of the fast component could also be elicited by exceptional stimulation such as a short-circuit recording in the unblocked state in Fig. 4.

The variation in maximum force reached at repeated contractions during drug administration is illustrated in Fig. 5 and 6 for C and dte, respectively. In the 5 experienced subjects similar smooth curves could be drawn using approximately all force determinations as estimates of MVC. But in the inexperienced subject this was not possible, and his data were therefore not used in this part of the study. Occasionally recovery MVC exceeded MVC recorded before drug administration (Fig. 5 one leg values).

Ratios between two and one leg extension forces were similar before and after recovery from drug administration (Table I). The average was used as control value. These ratios were reproducible from day to day. However two leg maximum force was relatively more reduced during administration of C than one leg maximum force. dte reduced one and two leg maximum forces to approximately the same extent. Two examples with particularly large falls in force development are shown in Fig. 5 and 6. During maximum effect of C

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The tests to maximum force observed here for the fast and slow components of force development are in the same order of magnitude as that noted during tetani of fast and slow motor units (Sitz 1963, Close 1967). In the present study the distributions of the tests to maximum force were skewed towards the right indicating an overrepresentation of slow elements within both components. If the subjects used an effort less than maximal at onset of contraction e.g. the uncurarized MVC recording of Fig. 3, this will tend to delay the rise in maximum force and thereby obscure the two component pattern. In general, the force pattern recorded by us during blocking supports the concept, that a fast and slow type of human muscle fibres work together during voluntary contraction. It also confirms earlier findings of the specificity of a blocking by dTC and C_{10} of human type I and II muscle fibres, respectively (Molbech and Johansen 1969, Bonde Petersen *et al.* 1975).

The fast component initiates the contractions recorded. The data of Grimby and Henneman (1968) suggested a change in the recruitment pattern in human skeletal muscle during contraction of high intensity (i.e. high velocity). The present data suggest that a partial change in muscle fibre recruitment takes place during intensive muscular contractions because fatigue in the fast contracting fibres appears rapidly. However the MVC recordings during C_{10} blocking show that slow fibres also can be active at onset of movement. The fast contracting element only reappears with renewed effort. This may explain the increase in the recorded strength after a shot or a shout as described by Ikai and Yoshida (1961).

Henneman *et al.* (1976) saw a significant correlation between force and type II muscle fibre area only at high contraction velocities. The present recordings support the finding that the fast contracting fibres are of importance for the rate of rise of tension rather than for the absolute force reached.

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group was performed in decerebrated cats. At maximal effort cortex cerebri might modify the function of the motor neurons. It was noted by Agnew and Preston (1965) that cortical stimulation of a pyramidal preparation resulted in either facilitation or inhibition of some motor neurons within a population innervating the same muscle, while both inhibitory and facilitatory influence was seen in others. It is in line with the present results that cortical stimulation in a similar preparation (Preston and Whitlock 1963) facilitates monosynaptic reflexes over the 'white' gastrocnemius, but inhibits reflexes over the 'red' soleus muscle. Thus, the neurologic component of the training response conforms to a decrease in cortical inhibition of type I motor units.

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A study of permanent adipsia induced by medial forebrain lesions

By

MATS RUDENORÉN and FRÅN FYHRQVIST

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Abstract

RUDENORÉN, M. and F. FYHRQVIST *A study of permanent adipsia induced by medial forebrain lesions.* Acta physiol. scand. 1978. 103. 463-471.

Cortical lesions involving most of the anterior wall of the third ventricle, and the medial part of the septal area, induced permanent loss of thirst in two goats. The ventral part of the basilar telencephalon remained intact in one of the animals. Pronounced dehydration (10-13% loss of b.wt.) developed during periods 0-7 days) when water supplementation was omitted. Determinations of plasma arginine vasopressin in one of the animals revealed that the dehydration did not cause any significant increase in the secretion of antidiuretic hormone. However the water deficit induced considerable rise in plasma renin activity and tachycardia. If anything, the carotid blood pressure became slightly elevated towards the end of 7 d dehydration periods. The lesions obviously inactivated cerebral sensory mechanism controlling water balance. It may have been due mainly to destruction of juxtaventricular receptors in the anterior hypothalamus region, but perhaps also to disruption of afferents from such receptors located posterior to this neural level.

Previous ablation studies have revealed that structures in, or close to the frontal wall of the third cerebral ventricle are essential for the maintenance of body fluid homeostasis (Andersson, Lehaell and Lishajko 1975; Buggy and Johnson 1977). Medially placed forebrain lesions which involved the entire anterior wall of the third ventricle were observed to cause a complete and persistent absence of the urge to drink in the goat (Andersson *et al.* 1975). The regulation of antidiuretic hormone (ADH) secretion was apparently also impaired in the adipsic animals, since very little antidiuretic activity could be detected in the urine when the goats were severely dehydrated. With the use of a sensitive radioimmunoassay method (Fyhrqvist, Wallenius and Hollemans 1976 b) it has recently been shown that moderate dehydration, leading to 5% elevation of plasma [Na⁺] causes roughly a 5-fold increase in

the plasma ADH concentration of normal goats (Olsson *et al.* 1978). This made it of interest to study with the same method, the ADH release in response to dehydration in the goat made adipic by medial forebrain damage. Since activation of the renin-angiotensin system may play a role in the regulation of the ADH-secretion (*cf.* Severs and Daniel-Severs 1973) it was also of interest to make parallel determinations of plasma renin activity (PRA). A further aim of the present study was to obtain if possible, more information about the extent of forebrain damage necessary to induce adipia.

Methods

Animals. Two female goats (in the following named I and II) were used. The animals (pre-lesioning b. w. 32 and 48 kg) were kept in metabolism cages (separating urine from feces) at room temperature ($20 \pm 1^\circ\text{C}$), where they had free access to hay and water. To maintain the goats in positive sodium balance they were each afternoon given 6 g of NaCl dissolved in 200 ml of tepid water. During the entire observation period both goats drank this salt solution voluntarily. The 4 h water intake and urine output were measured around 8 a.m. when samples of urine were taken for analyses.

Electrode implantation and lesioning technique. Both animals had a pair of thermocouple electrodes (o.d. 0.7 mm) permanently implanted into the preoptic/anterior hypothalamic region. The implantations were performed under general anesthesia (nembutal) 12 (Goat I) and 4 (Goat II) weeks before the electrodes were used for production of radio-frequency (RF) lesions. Goat I had the electrodes placed along the midline of the brain with an interspace of 4 mm, and the other animal had the electrodes bilaterally implanted 3 mm apart. The length of the uninsulated ends of the electrodes was 5 and 3 mm respectively. Coagulative lesions were made by applying RF energy between the thermocouple electrodes as previously described in detail (Andersson *et al.* 1975).

Water supplementation and hydration. Since the brain damage induced adipia, a daily water supplementation was administered by stomach tube to the rumen during the post-lesioning observation period. The amount of water (38°C) was adjusted to maintain roughly normal body weight and plasma [Na]. The water supplementation was omitted during dehydration tests. On some occasions an additional load of water (75 or 150 ml/kg b.wt.) was given in order to test the animal's ability to eliminate excessive body water.

Rectal sampling and analyses. To obtain clean urine samples during dehydration and hydration experiments, urine was collected via a Foley catheter. Jugular vein blood samples (10 ml) were drawn into heparinized syringes. For determination of the hematocrit (Ht) the blood samples were centrifuged in graded tubes at 3 000 rpm for exactly 7 min. Urine and plasma [Na] and [K] were measured by use of an "TL 343" internal standard flame photometer. An Advanced Instruments Inc. osmometer was used for determination of water and urine osmolality.

Hormone analyses. Ice-chilled syringes with 0.3 ml EDTA as anticoagulant were used to obtain blood samples for radioimmunoassays. The blood was immediately centrifuged at $+4^\circ\text{C}$, and the plasma was separated and stored at -20°C until radioimmunoassayed. Determinations of plasma arginine vasopressin (AVP) were performed with a method previously described (Fyhrqvist *et al.* 1976 b). The sensitivity of this method is 0.2 pg/ml. Radioimmunoassays of plasma renin activity (PRA) were made according to Fyhrqvist *et al.* (1976 a).

Blood pressure and heart rate recordings. Both animals had a polyvinyl catheter permanently implanted via the superficial temporal artery into the carotid artery as described by Eriksson, Fernández and Olsson (1971). The systolic/diastolic and the mean carotid blood pressures were recorded on an ml-writing polygraph via a Statham pressure transducer. When not registered during blood pressure recording, the heart rate was determined at intervals by auscultation.

Histology. At the end of the post lesioning observation period the animals were decapitated under nembutal anesthesia. The heads were perfused with isotonic saline followed by formalin/saline. After formalin fixation, a block of the brain (including the preoptic region, the hypothalamus and the thalamus) was embedded in celloidine and cut at 30 μ m by serial transverse (Goat I), or sagittal (Goat II) sectioning. The sections were stained either with toluidine blue, or according to Loyez (*cf.* Culling 1957) with several red as counter-staining.

Figures followed by \pm represent mean \pm S.E. Student's *t* test was used for statistical evaluations.

Results

I. Pre-lesioning studies

Intake studies

During the period between electrode implantation and lesioning the daily water intake remained rather stable in both goats (40–45 ml/kg). The urine output was 60–70% of the water intake and the urine osmolality varied between 700 and 1 700 mosm/kg. Great variations in renal excretion of Na⁺ (25–250 mmol/day) and K⁺ (60–230 mmol/day) were seen. Blood samples taken repeatedly during the pre-lesioning observation periods revealed that the blood plasma composition remained within the range earlier found in water replete normal goats ([Na⁺] 151.4 ± 0.6 mmol/l, [K⁺] 4.2 ± 0.1 mmol/l, osmolality 295 ± 2 mosm/kg) (Rundgren and Fjörquist 1978). The Ht was within the range 29 to 36. Pre-lesioning plasma AVP and PRA were only determined once in Goat II. The values obtained were AVP 3.1 pg/ml and PRA 1.19 ng/ml hr⁻¹.

Thermal and electrical stimulation

During the pre-lesioning observation periods the animals were repeatedly subjected to either moderate local forebrain warming (Goat I), or to bipolar electrical stimulation (Goat II) via the permanently implanted thermocouple electrodes. In Goat I the forebrain heating induced delayed drinking, and post-stimulatory hyperdipsia was observed in Goat II. These experiments have been described in detail previously (Rundgren 1978).

II. Post-lesioning studies

Acute effects

Both animals temporarily reacted with polyphasic panting to the RF coagulation of forebrain tissue. Goat I also showed 5-fold increase in water intake during the first post-lesioning 24 h period. No water diuresis developed in response to this over-hydration which caused considerable hemodilution (fall in plasma [Na⁺] to 132 mmol/l and plasma osmolality to 262 mosm/kg). No acute dipsogenic effect was observed in Goat II, which became adipsic immediately upon lesioning.

Chronic effects

Adipsia. Apart from the acute over-drinking seen in Goat I, none of the goats drank water spontaneously during the 90 and 93 days they were observed after lesioning. However both animals always eagerly drank the 200 ml of 3% NaCl solution they were offered daily as extra salt supply. On five (Goat I) and seven (Goat II) occasions the regular water supplementation was interrupted for 3 to 7 days. For ethical reasons, these dehydration periods were always terminated when the goats showed a tendency to reduce their food intake, i.e. when the water deficit had caused 10 to 13% reduction of the body weight. Peak plasma values at this degree of dehydration were [Na⁺] 183 mmol/l, [K⁺] 5.5 mmol/l and osmolality 395 mosm/kg, and the Ht had increased by about 40%. Apart from the complete lack of an urge to drink water and the slight reduction of appetite, the goats behaved normally when dehydrated to this extent.

the plasma ADH concentration of normal goats (Olsson *et al.* 1978). This made it of interest to study with the same method the ADH release in response to dehydration in the goat made adipic by medial forebrain damage. Since activation of the renin-angiotensin system may play a role in the regulation of the ADH-secretion (*cf.* Severs and Daniel-Severs 1973) it was also of interest to make parallel determinations of plasma renin activity (PRA). A further aim of the present study was to obtain, if possible, more information about the extent of forebrain damage necessary to induce adipia.

Methods

Animals. Two female goats (in the followi g named I and II) were used. The animals (pre-lesioning b. t. 32 and 28 kg) were kept in metabolism cages (separating urine from feces) in room temperature ($20 \pm 1^\circ\text{C}$) where they had free access to hay and water. To maintain the goats in positive sodium balance they were each afternoon given 6 g of NaCl dissolved in 200 ml of tepid water. During the entire observation period both goats drank this salt solution voluntarily. The 24 h water intake and urine output are measured round 8 a.m. when samples of urine were taken for analyses.

Electrode implantation and lesioning technique. Both animals had a pair of thermocouple electrodes (o.d. 0.7 mm) permanently implanted into the preoptic/anterior hypothalamic region. The implantations were performed under general anesthesia (nervobutal) 12 (Goat I) and 4 (Goat II) weeks before the electrodes were used for production of radio-frequency (RF) lesions. Goat I had the electrodes placed along the midline of the brain with an interspace of 4 mm, and the other animal had the electrodes bilaterally implanted 3 mm apart. The length of the uninsulated ends of the electrodes was 5 and 3 mm respectively. Coagulation lesions were made by applying RF energy between the thermocouple electrodes as previously described in detail (Anderson *et al.* 1975).

Water supplementation and hydration. Since the brain damage reduced adipia, a daily water supplementation was administered by stomach tube into the rumen during the post-lesioning observation period. The amount of water (38°C) was adjusted to maintain roughly normal body weight and plasma [Na⁺]. The water supplementation was omitted during dehydration tests. On some occasions an additional load of water (75 or 150 ml/kg b.wt.) was given in order to test the animal's ability to eliminate excessive body water.

Routine sampling and analyses. To obtain clean urine samples during dehydration and hydration experiments, urine was collected via a Foley catheter. Jugular vein blood samples (10 ml) were drawn into heparinized syringes. For determination of the hematocrit (Ht) the blood samples were centrifuged in graded tubes at 3 000 rpm for exactly 7 min. Urine and plasma [N⁺] and [K⁺] were measured by use of an IL 343[™] internal standard flame photometer. An Advanced Instruments Inc. osmometer was used for determination of plasma and urine osmolality.

Hormone analyses. Ice-chilled syringes with 0.3 M EDTA as anticoagulant were used to obtain blood samples for radioimmunoassays. The blood was immediately centrifuged at $+4^\circ\text{C}$, and the plasma was separated and stored at -20°C until radioimmunoassayed. Determinations of plasma arginine vasopressin (AVP) were performed with a method previously described (Fyhrquist *et al.* 1976 b). The sensitivity of this method is 0.2 pg/ml. Radioimmunoassays of plasma renin activity (PRA) were made according to Fyhrquist *et al.* (1976 a).

Blood pressure and heart rate recordings. Both animals had a polyvinyl catheter permanently implanted via the superficial temporal artery into the carotid artery as described by Eriksson, Fernández and Olsson (1971). The systolic/diastolic and the mean carotid blood pressures were recorded on an ink writing polygraph via a Statham pressure transducer. When not registered during blood pressure recording, the heart rate was determined at intervals by auscultation.

Histology. At the end of the post-lesioning observation period the animals were decapitated under nembutal anesthesia. The heads were perfused with isotonic saline followed by formalin/saline. After formalin fixation, a block of the brain (including the preoptic region, the hypothalamus and the thalamus) was embedded in celloidine and cut at 30μ by serial transverse (Goat I), or sagittal (Goat II) sectioning. The sections were stained either with toluidine blue, or according to Loyez (*cf.* Colling 1957) with neutral red as counter-staining.

Figures followed by \pm represent mean \pm S.E. Student's *t*-test was used for statistical evaluations.

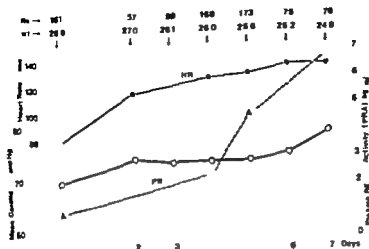


Fig. 2. Hemodynamic events and PRA during period when water supplementation was omitted in Goat II. Note the steep rise in PRA starting when the progressive ester loss had reduced 7% reduction of body weight. Plasma $[Na^+]$ expressed in mmol/l. B.P. = Blood pressure. B.Wt. = Body weight in kg. b/min = beats/min.

at a plasma $[Na^+]$ of 164 mmol/l. An appropriate reduction of renal water loss occurred during the dehydration periods, in spite of the fact that (according to the data obtained in Goat II) the ADH-secretion was not significantly increased. Thus, urine flow became reduced to roughly 0.1 ml/min with a concomitant rise in urinary osmolality to a level between 2000 and 2500 mosm/kg. This dehydration-induced reduction in urine flow was accompanied by a relatively low renal Na^+ excretion (10 to 50 μ mol/min).

The inability of Goat I to eliminate by a water diuresis the large amount of water consumed during the first 24 post-lesioning hours (see above) suggests that the forebrain lesioning induced an uncontrolled release of ADH. Also during later post-lesioning hydration tests the animal exhibited impaired ability to excrete excessive water. Thus, positive renal free water clearance was not seen until the water load had induced abnormal hemodilution (plasma $[Na^+]$ 135 mmol/l). As reported previously (Rundgren and Fyhrquist 1978) the presence of post-lesioning inappropriate ADH-secretion was even more obvious in Goat II, and as in that animal confirmed by plasma AVP determinations during hydration.

Hemodynamics and PRA The fact that both goats had completely lost the urge to drink after provided the possibility to study hemodynamic reactions to developing dehydration without the stressful interference of unsatisfied thirst. Fig. 2 illustrates the observed hemodynamic events, and PRA changes during a 7 d dehydration period in Goat II. As seen in this figure, the progressive hypovolemia developing during the dehydration periods was accompanied by an increase in heart rate. The tachycardia reached a plateau around 140 beats/min when the water deficit had induced about 10% reduction in body weight. The degree of dehydration induced in the present experiments (10 to 13% decrease in b.wt.) was in no case observed to cause any fall in the mean carotid blood pressure. Instead, slight increase in blood pressure was observed towards the end of the dehydration periods. At the same time PRA reached very high levels (Fig. 1 and 2). Therefore, pronounced activa-

TABLE I

	Pre-dehydration (n=8)	Moderate dehydration (n=8)	Pronounced dehydration (n=10)
Plasma [Na ⁺] (mmol/l)	150.0±0.8	155.0-164.9	165.0-175.0
Plasma osmolality (mosm/kg)	295.5±3	303-343	342-376
Plasma AVP (pg/ml)	3.90±0.68	3.35±0.39	5.17±1.01 ^a
PRA (ng/ml h ⁻¹)	1.27±0.29	2.44±0.30 ^b	3.73±0.48 ^a

Statistical significance from pre-dehydration values. N.S. ^a $p < 0.02$. ^b $p < 0.001$.
 Post lesioning plasma arginine vasopressin (AVP) and plasma renin activity (PRA) in Goat II during normal water balance, and at different degrees of water deficit during 6 dehydration periods.

ADH secretion. Determinations of plasma AVP were made in Goat II when the animal was supplemented with water to pre-lesioning body weight and plasma [Na⁺] and during 6 out of 7 dehydration periods. The values obtained in plasma [Na⁺] up to 175 mmol/l are presented in Table I. The AVP was not elevated during moderate dehydration (3 to 10% increase in plasma [Na⁺]), whereas a slight, statistically insignificant increase was observed during more pronounced dehydration (10 to 17% rise in plasma [Na⁺]). At the end of one of the dehydration periods, AVP was determined when the plasma [Na⁺] was as high as 182 mmol/l. A value of 7.7 pg/ml, i.e. twice mean pre-dehydration AVP concentration was observed. However on rehydration (Fig. 1) AVP returned to pre-dehydration level already

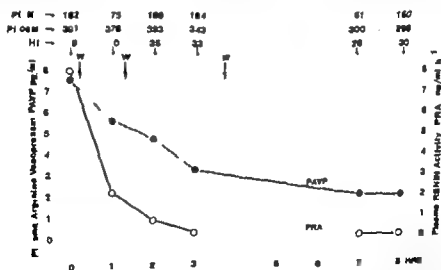


Fig. 1 The effect of gradual rehydration on plasma AVP and PRA in Goat II after 7 day period of omitted water supplementation which had induced pronounced dehydration. The elevation of AVP seen at the end of the dehydration period is only a fraction of that induced in the normal goat by 2 days of water deprivation (Olsson *et al.* 1978). Note that AVP returns to pre-dehydration level (see Table I) when plasma [Na⁺] and osmolality (—figures on top) remains considerably elevated above normal. W= Administration of 38°C water by stomach tube (100, 70 and 70 ml/kg respectively). Plasma [Na⁺] and osmolality on top of the figure are expressed in mmol/l and mosm/kg. Ht=hematocrit.



Fig. 4 A slightly oblique mid-sagittal section (30 μ) showing the extent of coagulative brain damage in Goat II (delineated by broken line). Arrow marked I indicates the anterior border of the lesion. E = electrode track. 3 Third ventricle; CC = Corpus callosum; CM = Corpus mammillare; CO = Choroid plexus; HYP = Anterior lobe of the hypophysis; MI = Mass intermedia; OV = Organ of vasculature. Stain. Mayer. Counter staining with neutral red explains the darkness of the anterior and the intermediate lobes of the hypophysis.

after, tonic inhibitory influence upon the release of ADH (*cf* Anderson 1977). However substantial increase in ADH-secretion as effect of hypovolemia does apparently not occur until the blood volume is reduced to such an extent that the arterial blood pressure falls (Doan *et al.* 1973 Arnold *et al.* 1977).

Among the supports for the idea that juxtaventricular receptors in the anterior hypothalamic region play an important role in the regulation of water intake and ADH-secretion are the results of ablation studies in the goat (Anderson *et al.* 1975) and the rat (Buggy and Johnson 1977). In both species, medially placed lesions destroying the whole anterior border of the third ventricle have been found to induce adipsia. To judge from bioassays of urinary antidiuretic activity in the goat, such lesions also impair ADH-secretion in response to dehydration. The latter observation has been confirmed and extended in the present study. Determinations of plasma AVP made in Goat II before, and at intervals after water supplementation had been omitted, revealed that a water deficit causing >10% increase in plasma [Na] did not cause any significant increase in the ADH-secretion (Table I), although the cerebral damage in this goat (Fig. 3 and 4) left the supraoptic and paraventricular nuclei intact. However a conspicuous rise in PRA was seen at this degree of dehydration. Since in normal goats dehydration to less than half that extent causes large increases in plasma AVP (Olsson *et al.* 1978), it suggests that the forebrain lesion in Goat II severely impaired a regulation of ADH-secretion which is dependent upon extracellular [Na] and, eventually upon activation of the renin-angiotensin system. The relatively small (about two-fold) increase in AVP observed during a water deficit elevating plasma [Na] to 182 mmol/l (Fig. 1) indicates that some residual regulation dependent upon extracellular [Na] and/or

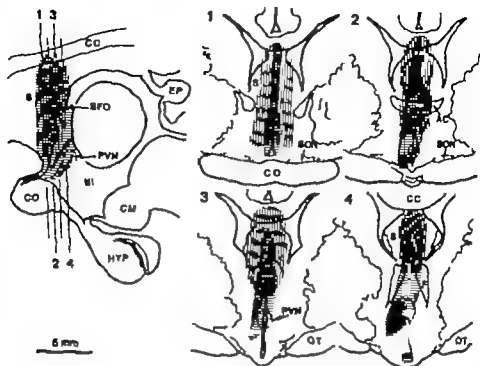


Fig. 1 A midsagittal section through the hypothalamic region of the goat (left), and four transverse sections (corresponding to the numbered levels on the sagittal section). Cerebral damage induced in Goat I horizontally hatched in Goat II vertically hatched III—Third ventricle AC—Anterior commissure CC—Corpus callosum CM—Corpus mammillare CO—Chiasma opticum EP—Epiphysis, F—Descending column of the fornix, HYP—Anterior lobe of the hypophysis, OT—Optic tract; PVN—Paraventricular nucleus, S—Septal region SFO—Subfornical organ SON—Suboptic nucleus.

tion of the renin-angiotensin system may have contributed to preserve, or even increase the blood pressure. On rehydration, the PRA rapidly returned to pre-dehydration, or even lower levels (Fig. 1).

Forebrain lesions The extent of the cerebral lesions in Goat I and II is projected on midsagittal and transverse sections in Fig. 3 (horizontally and vertically hatched areas, respectively). The brain damage in common (—crosshatched areas) destroyed most of the anterior wall of the third ventricle and the medial portion of the septal region. However a fraction of the antero-ventral border of the third ventricle (including most of the organum vasculosum of the lamina terminalis, and the supraoptic recess) was untouched by the lesion in Goat II (Fig. 4).

Discussion

Much experimental evidence favours the new concept that thirst and ADH-secretion in response to elevated extracellular $[Na^+]$ and osmolality are mediated by sodium sensitive receptors which predominantly are located close to the anterior wall of the third ventricle. The same cerebral sensory mechanism appears to become engaged also when hypovolemia-induced activation of the renin-angiotensin system elicits thirst and ADH release. In addition, impulses from cardiovascular distention receptors and baroreceptors seem to exert a

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angiotensin II might have been present. However, this elevation of AVP could also have been due to diminished cardiovascular inhibition of the ADH-secretion.

An uncompensated transient water diuresis, indicating a sudden elevation of the stimulus threshold for ADH release and thirst, has previously been observed as an acute effect of medially placed forebrain lesions (Andersson *et al* 1975), and lesions mainly confined to the septal region (Rundgren and Fyhrquist 1978). No such acute effect of lesioning was obtained in the present animals. The reason by all probability is that, in addition to adipsia and elevated dehydration threshold for ADH-release, the cerebral damage caused apparent inappropriate ADH-secretion. As discussed in a previous paper (Rundgren and Fyhrquist 1978), the latter effect may have been due to the fact that the lesions on one, or both sides, extended near the supraoptic nuclei (Fig. 3 transverse sections 1 and 3), and hereby interrupted pathways mediating reflex volumetric inhibition of the ADH release.

Although a persistent effect of the present forebrain damage was adipsia, one of the animals (Goat I) paradoxically exhibited transient hyperdipsia as acute effect of lesioning. A near at hand explanation is that lesioning in this animal temporarily caused irritative stimulation of "thirst-pathways" at the border of the region affected by the RF-heating. A non-specific stimulation of this kind might have occurred in the septal region, since here the permanent brain damage was much more narrow than in Goat II which did not drink at all after lesioning (Fig. 3 transverse section 1).

The ventral limitation of the cerebral damage in Goat II demonstrates that adipsia in fact can be induced by forebrain lesions leaving most of the organum vasculosum of the lamina terminalis intact. This has not become evident from previous ablation studies. It does not exclude the possibility that receptors involved in the control of water balance are located also in the most antero-ventral fraction of the ventricular wall, since the lesion might have interrupted pathways mediating receptor information from that area. Further more, the present study by no means disproves that juxtaventricular receptors regulating water intake and ADH-release also may be distributed posterior to the frontal border of the third ventricle. Afferents from receptors of that location could well have been disrupted by the forebrain lesions induced here. Of some interest with regard to this possibility is that infusions of hypertonic NaCl and angiotensin II into the fourth ventricle recently have been observed to cause apparent release of ADH in hydrated goats (L. G. Leckell and M. Rundgren, unpublished).

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Effects of hypoxia on the rat portal vein *in vitro* P_{O_2} gradients in tissue and surrounding fluid

By

PER HELLSTRAND

Myogenic spontaneous activity of the isolated rat portal vein decreases with lowered P_{O_2} of the bath medium (Hellstrand, Johansson and Norberg 1977). Graded inhibition occurs at $P_{O_2} < 50$ mmHg and is caused by depression both of electrical membrane activity and of energy supply to contraction. An anoxic core in the muscle at ambient P_{O_2} as high as 30-50 mmHg was not considered likely on estimation of the P_{O_2} drop within the muscle wall. Consequently it was suggested that hypoxia might influence the smooth muscle cells themselves in a graded way over a limited P_{O_2} range.

Recently Fay Nair and Whalen (1977), using micro- P_{O_2} -electrodes, have demonstrated a significant P_{O_2} drop near the surface of a guinea pig ductus arteriosus mounted *in vitro*, presumably due to an unstirred layer of fluid surrounding the preparation. The possibility arises that such an effect, leading to an anoxic core in the vessel wall, might explain the graded response of the portal vein to hypoxic solutions. In the present study this problem has been approached by mounting a conventional membrane-covered polarographic P_{O_2} electrode in direct contact with the surface of portal veins to get an estimate of the minimal P_{O_2} to which the smooth muscle cells are exposed. Simultaneously some observations could be made on the temporal relation between phasic contractions and oxygen consumption.

Portal veins were cut open longitudinally, spread out as rectangular plates (approx. 0.1-3.4 × 6-9 mm), and mounted horizontally between a micrometer and a Grass FT03 force transducer. An O₂ electrode located vertically above the strip could be lowered to make contact with its surface. The muscle and electrode were mounted in a 50 ml organ bath at 37°C filled with bubbled Krebs solution. After equilibration for about 45 min P_{O_2} was lowered in steps as previously described (Hellstrand *et al.* 1977). The polarographic O₂ electrode (Radiometer, Copenhagen, Denmark) had its tip shaped as a flat disc, large enough to cover the whole muscle sheet. Some experiments were run using an electrode with a half-spherical tip (L. Eachweiller & Co. Kiel, Germany) providing a smaller contact area with the muscle. No difference was apparent in the results obtained with the two electrodes.

Fig. 1 shows isometric spontaneous activity of a portal vein, its time integral and the \dot{V}_{O_2} electrode recording. At first the electrode tip is located in the bath fluid 5 mm above the strip. As it is lowered to touch the muscle (first arrow) P_{O_2} falls from 115 mmHg to around

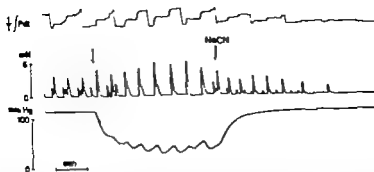


Fig. 1. Spontaneous activity of portal vein (middle trace), time integral of tension over 1 min periods, in arbitrary units (upper trace), and O_2 electrode recording (lower trace). P_{O_2} in bath medium 115 mmHg. First arrow electrode lowered 5 mm to make contact with muscle. Second arrow addition of 2 mM NaCN.

33 mmHg. It is seen that this surface P_{O_2} oscillates in pace with the spontaneous contractions. At the second arrow respiration of the muscle is blocked by addition of 2 mM NaCN. With the electrode still lowered, P_{O_2} returns to 115 mmHg. Thus, O_2 consumption by the electrode does not appreciably influence P_{O_2} recorded at the muscle surface. Even though contractions persist for a few minutes in the poisoned muscle there are no oscillations on the rising P_{O_2} curve (cf. the falling curve). This indicates that those seen in the unpoisoned muscle are not movement artifacts but signs of varying O_2 uptake. Neglecting electrode delay it was a consistent finding that surface P_{O_2} started to fall near the peak of contraction. Apparently the cell respiration responds very quickly to changes in contractile activity.

In Fig. 2 the integrated spontaneous activity recorded with the electrode lowered is plotted vs. solution P_{O_2} (filled circles) and vs. surface P_{O_2} (open circles), respectively (6 exps). At each P_{O_2} level spontaneous activity was integrated for 6 min. Solution P_{O_2} was recorded immediately before and after this period by raising the electrode 0.4–5 mm. In

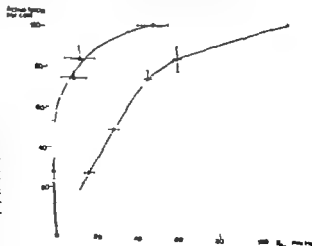


Fig. 2. Integrated spontaneous activity vs. P_{O_2} in bath (filled circles) and vs. P_{O_2} at muscle surface (open circles). 6 experiments. Means \pm SE shown. Curves drawn by eye. Integrated activity during control P_{O_2} (115 mmHg) set at 100%. All points refer to mechanical activity measured with electrode at muscle surface.

this range, solution P_{O_2} was independent of distance from the muscle. It is seen from the open circles that a dramatic reduction of activity occurs only when surface P_{O_2} is very low 0–2 mmHg. It must be remembered that in these experiments one surface only of the strip is exposed to bubbled solution. By symmetry then, the electrode can be thought of as measuring P_{O_2} in the centre of a hypothetical strip of double thickness (*i.e.* about 0.2 mm) oxygenated at both surfaces. The O_2 consumption of the portal vein is about $0.55 \mu\text{mol}/\text{min g}$ (Hellstrand 1977). The P_{O_2} drop from surface to centre of a 0.2 mm muscle strip can thus be estimated at 36 mmHg (*cf.* Pittman and Duling 1973). At control P_{O_2} in the bath, a difference of 60–70 mmHg between solution and surface P_{O_2} was observed (Fig. 1 and 2). Roughly half of this may thus be expected to be located outside the vessel wall, indicating the presence of an unstirred layer of about $100 \mu\text{m}$ in the solution close to the muscle (R. N. Pittman, personal communication). Assuming such a layer to exist also with no electrode present the P_{O_2} drop in the solution near the surfaces of a 0.1 mm strip can be calculated to 10–15 mmHg. With the above assumptions, the P_{O_2} drop within the strip is about 9 mmHg. The observed range of 40–50 mmHg for an effect of P_{O_2} on the muscle activity thus corresponds to 20–25 mmHg at the level of the innermost cells. Still, many estimates of "critical tissue P_{O_2} " indicate a value of only a few mmHg (Pittman and Duling 1973, Fay *et al.* 1977). A possible explanation for the difference might be, first, the oscillatory behaviour of the O_2 consumption noted in this study leading to a "part-time anoxia" for some of the cells, and, second, the effects of hypoxia on the electrophysiology of the portal vein. Since this smooth muscle carries propagated action potentials, hypoxic depolarisation of a pacemaker region will influence other parts of the preparation. These electrophysiological effects of hypoxia, are presently under study.

Valuable suggestions concerning effects of unstirred layers *in vitro* and the means to study them have been given by Drs B. R. Duling and R. N. Pittman. Miss M. Lundahl, M. Heidenholm and I. Nordström have given able technical assistance. The study was supported by the Swedish Medical Research Council (Grant 04X-00028), the Medical Faculty, University of Lund, and AB Hälsö, Göteborg.

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Enkephalin-like immunoreactivity in nerve terminals in sympathetic ganglia and adrenal medulla and in adrenal medullary gland cells

By

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LARS-GÖSTA ELFVÉN and ROBERT ELDE

Recently Hughes *et al.* (1975) have isolated and structurally identified two pentapeptides from the brain, the enkephalins, which may represent endogenous ligands for the opiate receptors (Terenius and Wahlström 1974, 1975; Hughes *et al.* 1975; Pasternak *et al.* 1975). Their distribution has been studied with biochemical and histochemical techniques both in the central (Simantov *et al.* 1976, Smith *et al.* 1976, Hong *et al.* 1977; Hughes *et al.* 1977; Elk *et al.* 1976, Hökfelt *et al.* 1977a, Simantov *et al.* 1977; Watson *et al.* 1977) and peripheral (Elde *et al.* 1976, Hughes *et al.* 1977) nervous system. In the present paper preliminary results on the distribution of enkephalin-like immunoreactivity in sympathetic ganglia and the adrenal gland of guinea pigs are reported.

Male guinea pigs (b.wt. 300-400 g) were anesthetized and perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer solution. After 30 min the inferior mesenteric ganglion and the adrenal gland were dissected out, immersed in the same fixative for at least 90 min, rinsed in sucrose containing buffer cut on a cryostat and processed for immunocytochemistry according to Coons and collaborators (see Coons 1958). The sections were incubated in a humid atmosphere for 24 h at +4°C with antiserum raised to methionine-enkephalin (met-ENK) conjugated with bovine serum albumin (BSA). The serum had been pretreated with BSA and a dilution of 1:160 was used. After rinsing in phosphate buffered saline (PBS), a second incubation was performed at +37°C for 30 min with fluorescein-isothiocyanate (FITC) conjugated antibodies (Statens Bakteriologiska Laboratorium, Stockholm, Sweden). The sections were mounted in glycerine-PBS (3:1) and examined in a Zeiss fluorescence microscope. Met-ENK antiserum pretreated with an excess of met-ENK (100 µg/ml antiserum diluted 1:160) served as a control serum.

In the inferior mesenteric ganglion a very dense plexus of ENK-immunoreactive, varicose nerve terminals was observed. Strands of several terminals surrounded the majority of the principal ganglion cells (Fig. 1). Occasionally small groups of principal ganglion cell profiles were seen which in single sections did not seem directly related to fluorescent fibers. No principal ganglion cells, but a few small intensely fluorescent (SIF) cells (see Erilinkö 1976) were met-ENK positive. No fluorescent fibers were observed around blood vessels.

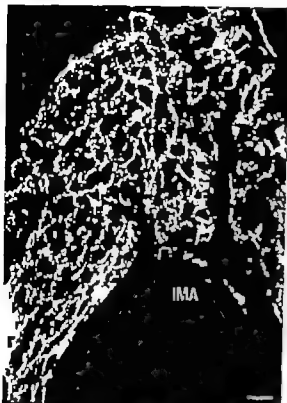


Fig. 1



Fig. 2

Fig. 1. Immunofluorescence micrograph of the inferior mesenteric ganglion after incubation with met-ENK antiserum. A dense plexus of immunoreactive nerve terminals is seen around the principal ganglion cells. IMA = inferior mesenteric artery. Ba indicates 50 μ m.

Fig. 2. Immunofluorescence micrograph of the adrenal gland after incubation with met-ENK antiserum. Strongly fluorescent gland cells and some fluorescent nerve terminals (arrows) are seen. Asterisks indicate adrenal cortex. Ba indicates 50 μ m.

In the *adrenal medulla* a large number of gland cells exhibited met-ENK immunoreactivity of varying intensity. Among the fluorescent gland cells a sparse network of varicose met-ENK positive nerve terminals could be seen (Fig. 2).

The present findings suggest that nerve terminals in some sympathetic ganglia and in the adrenal gland contain an enkephalin-like peptide. Other biologically active peptides, such as substance P and VIP have also been found here (Hökfelt *et al.* 1977 b, c). Thus, the peripheral autonomic nervous system may contain a further class of neurons, peptide neurons, in addition to classical cholinergic and adrenergic systems. Besides nerve terminals, a large proportion of gland cells of the adrenal medulla seems to contain the same or a similar peptide. This is another example of the presence of a biologically active peptide in amine-containing cells which is characteristic of so-called APUD (Amine content and/or amine Precursor Uptake and Decarboxylation) cells (Pearse 1969).

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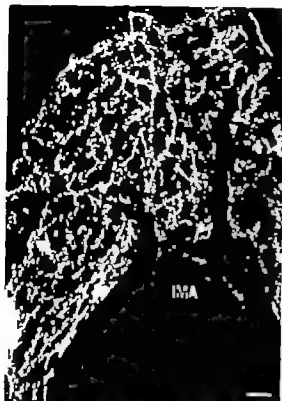


Fig. 1



Fig. 2

Fig. 1 Immunofluorescence micrograph of the inferior mesenteric ganglion after incubation with met ENK antiserum. A dense plexus of immunoreactive nerve terminals is seen around the principal ganglion cells. IMA = inferior mesenteric artery. Ba indicates 50 μ m.

Fig. 2 Immunofluorescence micrograph of the adrenal gland after incubation with met ENK antiserum. Strongly fluorescent gland cells and some fluorescent nerve terminals (arrows) are seen. Asterisks indicate adrenal cortex. Bar indicates 50 μ m.

In the *adrenal medulla* a large number of gland cells exhibited met ENK immunoreactivity of varying intensity. Among the fluorescent gland cells a sparse network of varicose met ENK positive nerve terminals could be seen (Fig. 2).

The present findings suggest that nerve terminals in some sympathetic ganglia and in the adrenal gland contain an enkephalin-like peptide. Other biologically active peptides, such as substance P and VIP have also been found here (Hökfelt *et al.* 1977 b, c). Thus, the peripheral autonomic nervous system may contain a further class of neurons, peptide neurons, in addition to classical cholinergic and adrenergic systems. Besides nerve terminals, a large proportion of gland cells of the adrenal medulla seems to contain the same or a similar peptide. This is another example of the presence of a biologically active peptide in amine containing cells which is characteristic of so-called APUD (Amine content and/or amine Precursor Uptake and Decarboxylation) cells (Pearse 1969).

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Failure of somatostatin to eliminate the glucagon release induced by baroreceptor unloading in the cat

By

JOHANNES JÄRHULT BO ÅHRÉN, MATS ERICSSON and JENS HOLST

Several studies have demonstrated that somatostatin can suppress the release of pancreatic glucagon both in animals and man. Thus, an infusion of somatostatin lowers the basal glucagon level (see Luft *et al* 1978) and somatostatin almost totally blocks the stimulatory effect on the glucagon release induced by insulin hypoglycemia (Chideckel *et al* 1975), arginine (Gerich *et al* 1974), adrenaline (Weir *et al* 1974), orciprenaline (Raptis *et al* 1977) or isoproterenol (Gerich *et al* 1975). It has been shown recently by our group that large amounts of glucagon are reflexly released into the portal circulation in response to unloading of the carotid baroreceptors in the cat (Järhult and Holst 1977, 1978). The present experiments were performed to determine if somatostatin also eliminates this stimulatory effect on the glucagon release.

Methods. Four cats (mean weight 2.5 kg) were anesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg) after induction with ether. A tracheal cannula was inserted. The vagus nerves and the carotid arteries were dissected free in the neck. After heparinization, polyethylene catheters were placed in the right femoral artery, the portal vein and in the right aortic vein. The vagus nerves were cut in the neck and about 30 min later a 1-l infusion of synthetic cyclic somatostatin (Serono 50 µg/h) was started. After 30 min of somatostatin infusion, the carotid baroreceptors were unloaded for 45 min by clamping the bilateral common carotid arteries. Portal blood samples for determination of immunoreactive glucagon and insulin and arterial blood samples for determination of glucose were drawn at intervals before and during the somatostatin infusion. Portal plasma glucagon concentration was determined by means of a recently described radioimmunoassay technique (Holst *et al* 1976), using an antiserum which is highly specific for pancreatic glucagon. Portal plasma insulin concentration was measured according to Albano *et al* (1972). Arterial plasma glucose concentration was measured with the glucose-oxidase method.

Results. Fig. 1 illustrates that somatostatin lowered the basal glucagon and insulin levels in portal plasma. However, somatostatin could not prevent a large increase occurring in the portal plasma glucagon concentration (IRG) in response to the baroreceptor unloading (-carotid occlusion). IRG thus rapidly rose from 102 ± 31 pg/ml to 256 ± 43 pg/ml in response to carotid occlusion and this level was roughly maintained until the clamps were removed, when IRG promptly returned to its pre-occlusion level. Carotid occlusion could not further depress the somatostatin-induced lowering of the portal plasma insulin concentration (IRI). Somatostatin did not change the basal arterial plasma glucose concentration, which rose by about 4 mM upon carotid occlusion.

Fig. 2 shows a comparison between the changes of IRG in response to the unloading of the carotid baroreceptors with or without somatostatin infusion. Panel A illustrates that,

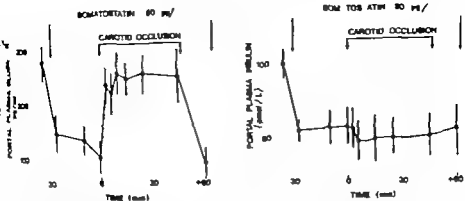


Fig. 1. Changes of portal glucagon-reactive glucagon (left) and of insulin concentrations (right) in response to unloading of the carotid baroreceptors (= carotid occlusion) during somatostatin infusion in 4 cats. Mean values \pm S.E. are given.

Carotid occlusion led to a more pronounced increase in IRG in normal cats than in somatostatin-treated cats. It should be noted, however, that the pre-stimulatory glucagon concentration was 3 times higher in the intact cats than in the somatostatin treated cats and the same changes in IRG are calculated in percentage, there was no statistically significant difference between the two groups of animals (Panel B).

Comments. Several previous studies have demonstrated that somatostatin lowers the basal plasma glucagon level and that it blocks the stimulatory action of different secretagogues on the A-cell. These findings have supported the hypothesis that somatostatin may interfere with a late step in the hormone-releasing mechanism common for all secretagogues, e.g. the calcium dynamics. However, the present results suggest that glucagon might be released by some stimulus which is not completely inhibited by somatostatin. The exact mechanism(s) by which glucagon is released in response to baroreceptor unloading is not known, but the reflex is at least partly dependent on the sympatho-adrenal system, since the hyperglucagonemia is substantially reduced after cutting the splanchnic nerves (Djavanmard and Holst 1978). An interference by somatostatin with adrenergic mechanisms has been suggested by Smith *et al.* (1977) who found that the somatostatin-mediated inhibition of basal glucagon release was prevented by blockade of the α -adrenoceptors. A link between somatostatin and the sympatho-adrenal system with regard to endocrine secretors is also supported by Jirhult and Lundquist who observed that somatostatin can be released from the cat's pancreas in response to sympathetic stimulation (unpublished results). The dose of somatostatin which has been used in this study (20–25 μ g/kg/h) is somewhat higher than doses which have earlier been found to effectively suppress the glucagon release. For instance, Gench *et al.* (1974) found that 20 μ g/kg/h of somatostatin completely abolished the arginine-induced glucagon secretion in man. We therefore consider it unlikely that the increase in IRG upon carotid occlusion could be caused by too low a dose of somatostatin. Furthermore, the dose was sufficient for a marked reduction of the basal glucagon and insulin levels. It is therefore suggested that the glucagon release in response to baroreceptor unloading might be partly linked to some, yet unidentified, somatostatin-insensitive releasing mechanism.

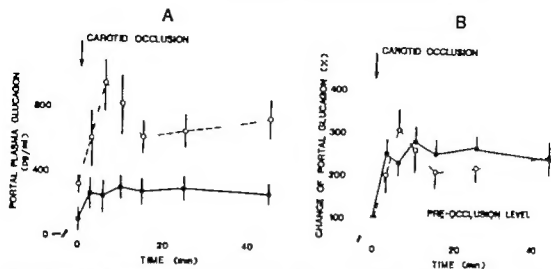


Fig. 2. Changes of portal plasma immuno-reactive glucagon concentration during unloading of carotid baroreceptors in cats during (●—●) or without (○—○) somatostatin infusion in cats. Results from intact cats have been calculated from previous published data (Järhult and Holst 1978). The changes in absolute terms are presented in Panel A, whereas Panel B depicts the changes in percent of the pre-occlusion value. Mean values \pm S.E. are given.

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or Communications:

- HELLSTRAND, P. Effects of hypoxia on the rat portal vein *in vitro*. P gradients in time and surrounding fluid
- SCHULTZBERG, M., T HÖKFELT J M. LUNDBERG, L. TERENTIUS, L.-G. ELFVIN and B. ELDE. Enkephalin-like immunoreactivity in nerve terminals in sympathetic ganglia and adrenal medulla and in adrenal medullary gland cells
- JÄRHULT J., B. AHRÉN, M. ERICSSON and J. HOLST Failure of somatostatin to enhance the glucagon release induced by baroreceptor unloading in the cat

Supplement appended.

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Figures should not be larger than manuscript pages and sent in as glossy prints in a size required for reproduction. Lettering should be large enough to permit suitable reduction and print uniform size. When possible, diagrams and photomicrographs should extend horizontally vertically in order to save space. Photomicrographs should be calibrated on the print (not as factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum, both in number and size, with text above the table (not on sheets). Single numbers in a series should be replaced by mean and S.D. or mean and S.E., in the 1 with number of observations.

Key words (5-10) are recommended in order to facilitate indexing.

For abbreviations, units, and symbols see special list in the Journal and recent articles.

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The International system of units (SI)

The following symbols and units, recommended by the SI, are being used in *Acta Physiologica*. Certain units, not included in SI, will still be permitted.

SI units with recommended symbols

Units	Symbols
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol μ mol nmol pmol
meter millimeter	m mm
micrometer	μ m
nanometer	nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N ($kg \cdot m/s^2$)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J ($N \cdot m$)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Permitted non-SI units

Units	Symbols
gramme	g
millimetre	mm
liter	l
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)
curie	Ci
liter milliliter microliter	l ml μ l
degree Celsius	$^{\circ}$ C

Conversion factors to be given in Methods.

